SYNERGISTIC EFFECT BETWEEN HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOPOIETIN-1 COMBINED WITH MYOBLAST THERAPY FOR TREATMENT OF ISCHEMICALLY DAMAGED HIND LIMBS

NIAGARA MUHAMMAD IDRIS

(B.App.Sc (Hons), NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF SURGERY

NATIONAL UNIVERSITY OF SINGAPORE

2004

ACKNOWLEDGEMENTS

My sincerest thanks to A/P Eugene Sim for giving me the invaluable opportunity to engage in this project, for all the advice shared throughout the study and for the professional training on presenting my work at conferences, without which I would not be 'self-pushed'. Exceptional thanks go out to Dr Khawaja Husnain Haider who has been a mentor, father, brother and friend to me. Your guidance, support and understanding, especially during trying times, are deeply appreciated. To our doctor lab mates - Ye Lei, Jiang Shujia, Li Wei and Guo Changfa, your generosity and willingness to share pointers on surgical techniques are highly regarded. Special regards for Ye Lei and Valerie Koh who have countless times gave me the extra technical and intellectual support in this project. And to other fellow lab members – Rufaihah Abdul Jalil, Michelle Goh and Siti Saifa Hussain - who have helped me in one way or another, your assistance and warm acceptance, are truly valued.

Many thanks to A/P Lim Yean Teng, Dr Poh Kian Keong and members of the Catheterization Laboratory, National University Hospital, for technical assistance in the angiographic study.

Special acknowledgement to Dr Leslie Retnam, Dr Enoka Bandularatne, and members of Animal Holding Unit, NUS, for giving me expertise advice and technical support with regards to animal work.

I would also like to acknowledge and thank Dr Ge Ruowen and Dr Winston Shim from Department of Biological Sciences, NUS, for supplying us with the adenoviral vectors.

Thanks to Prof Peter K. Law and Dr Florence Chua from Cell Transplants Singapore Pte. Ltd. for providing us with the patented Supermedium.

And not to forget friends and family members who have been supportive and encouraging throughout this enriching period of my life.

The work was carried out at National University of Singapore and National University Hospital, and financially supported by National Medical Research Council (Singapore) Grant # R-364-000-017-213 and IBG-NUMI Cardiovascular Research Program R364-000-021-213.

TABLE OF CONTENTS

SUMMARY

Therapeutic angiogenesis aimed at improving peripheral blood flow by stimulating the growth and development of collateral vessels has been proposed as a potential treatment for peripheral vascular disease. The strategy is based on bio-bypassing the under-perfused tissues by inducing the formation of new blood vessels through administration of angiogenic growth factors. However, many have relied on single angiogenic growth factor protein or its gene administration, which proved to be insufficient for the development of a functional vascular system. Synergistic interaction between two of these factors – vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) - to produce functional leakage-resistant neovascularization has been proposed as a promising and safe candidate for therapeutic angiogenesis. Combining the synergistic effect of $VEGF_{165}$ and Ang-1 together with myoblast (muscle precursor cells) therapy for treatment of ischemic limb disease would have the additional advantage of inducing myogenesis on top of the commonly reported angiogenesis in ischemic limbs.

In the present study, rabbit autologous primary skeletal myoblasts were used as carriers of a novel bicistronic adenoviral vector simultaneously expressing VEGF and Ang-1 genes. The cells were prior labeled with retrovirally transduced LacZ reporter gene, 4,6-diamidino-2-phenylindole (DAPI) and 5-bromo-2'-deoxy-uridine (BrdU) before transplanted into a rabbit model of hind limb ischemia. Approximately eight weeks after treatment, neovascularization in the limb was assessed by angiography and the animals were euthanized and harvested for histological studies.

Extensive transplanted skeletal myoblast survival was observed in all cell-transplanted groups as visualized by DAPI, BrdU and X-gal staining. Angiographic blood vessel count revealed enhanced neovascularization in the group that received transplantation of myoblasts overexpressing VEGF and Ang-1 as compared to other control groups. This was confirmed with immunohistochemical staining for von Willebrand Factor-VIII which consistently demonstrated a significant increase in capillary density of this group as compared to control groups.

The study therefore revealed that simultaneous expression of VEGF and Ang-1 from bicistronic adenoviral vector transduced skeletal myoblasts potently stimulated enhanced functional neovascularization in the ischemic limbs.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ad	Adenovirus
Ad-Bicis	Adenovirus carrying both $hVEGF_{165}$ and hAng-1 genes
Ad-Null	Adenovirus without angiogenic gene
Ad-VEGF	Adenovirus carrying only $hVEGF_{165}$ gene
AEC	3-amino-9-ethylcarbazole
aFGF	Acidic fibroblast growth factor
Ang-1	Angiopoietin-1
bFGF	Basic fibroblast growth factor
BrdU	5-bromo-2'-deoxy-uridine
BSA	Bovine serum albumin
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
E1	Early region 1
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flt-1/VEGF-R1	*fms*-like tyrosine kinase-1
HRP	Horse radish peroxidase
IRES	Internal ribosome entry site
KDR/Flk-1/VEGF-R2	Kinase insert domain-containing receptor/fetal liver kinase-1

MMLV	Moloney Murine Leukemia Virus
MyHC	Myosin heavy chain
Myo	Myoblasts
*nls*LacZ	LacZ reporter gene with nuclear localization signal
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PVD	Peripheral vascular disease
SMA	Smooth muscle actin
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor VIII
X-gal	5-bromo-4-chloro-3indoyl-β-D- galactopyranosid

1 INTRODUCTION

1.1 Peripheral vascular disease

Peripheral vascular disease (PVD) is often caused by atherosclerotic narrowing of the arterial lumen. The gradual deposits of lipids, cholesterol, calcium, smooth muscle cells and platelets in the atherosclerotic plaque along the artery walls restrict the flow of blood, having the most negative impact typically on the legs and feet. The reduction in blood flow is often insufficient for supplying oxygen and nutrients to meet the metabolic demands of exercising leg muscles, thereby resulting in a condition of leg pain, termed 'intermittent claudication'. In severe cases, it can result in critical limb ischemia. By then, pain at rest, non-healing wounds and gangrene (dead tissue) are common unavoidable consequences. If left untreated, these progressive gangrene, rapidly enlarging wounds or continuous ischemic rest pain can be a threat to limb viability.

Peripheral vascular disease is a common condition with variable morbidity affecting mostly population older than 50 years of age. With approximately 5 % having intermittent claudication and another 1 % suffering from critical limb ischemia (reviewed in Halperin, 2002), it is an increasingly common problem in developed countries, including Singapore.

Currently, therapeutic options to treat PVD include medical therapy, non-surgical and surgical interventions. While medications do not eliminate the narrowing of arteries, it is prescribed in order to improve blood flow and relieve symptoms. It is used alone or in combination with one of the other treatments. To treat plaque accumulation by either

removing, compressing or displacing it, non-surgical interventions that involve minimally invasive interventional procedures such as angioplasty, sometimes followed by stent implantation, atherectomy and thrombolytic therapy provide alternative treatment modalities. However, in the case of severe symptoms that do not respond to any of these treatment forms, surgical interventions such as bypass surgery may be necessary. This involves bypassing the diseased part of the artery by creating new channels to redirect blood circulation around the site of the arterial block. Unfortunately, bypass surgery is ineffective if the blockage or narrowing in the artery is too extensive, or if the vessel is too small, leaving many patients with lower limb amputation as the sole therapeutic option. This disease is also associated with significant mortality, with life expectancy in 30 % of patients being less than two years after their first major amputation. The primary cause of mortality in these patients is myocardial infarction, stroke and complications from diabetes (Coffman, 1991). Indeed, psychological testing of these patients has typically revealed quality-of-life indices similar to those of patients with cancer in critical or even terminal phases of their illness (Albers et al., 1992).

Despite major advances in these areas, PVD is a challenging disease and difficult to manage with the current treatment modalities. In some cases, even short-term results in treated patients with advanced stage disease remained unsatisfactory. Indeed, there is a great clinical need for new treatment options that may stimulate collateral blood vessel growth, increase vascularity and improve skeletal muscle function that would subsequently translate to substantial improvements in mobility, exercise tolerance, and quality of life.

1.2 Therapeutic angiogenesis

Pioneering work of Folkman et al. (1987) and Tomasi et al. (1990) on isolation and description of a number of angiogenic growth factors for therapeutic angiogenesis aimed at improving peripheral blood flow by stimulating the growth and development of collateral vessels, have paved the way for a potentially useful alternative treatment for PVD (Fox et al., 1996; Isner et al., 1996a, b; Taniyama et al., 2001; Vale et al., 2001; Silvestre and Levy, 2002). The strategy is based on bio-bypassing the under-perfused tissues by inducing the formation of new blood vessels (angiogenesis) and has been attempted by using various angiogenic growth factors (Hayashi et al., 1999; Gowdak et al., 2000a, b; Vincent et al., 2000; Kyriakides et al., 2001; Ohara et al., 2001; Morishita et al., 2002; Shimpo et al., 2002). These factors are naturally expressed to initiate the growth of collateral vessels in regions where blood flow is limited as a reparative mechanism. However, an imbalance between the rapidity and extent of 'natural' collateral vessel growth versus the progression of the occlusive arterial disease hinders the recovery process. An outside intervention is needed to supplement the inadequate intrinsic repair mechanism through the delivery of exogenous angiogenic growth factors (Melillo et al., 1997). More recently, implantation of stem cells has been carried out together with the delivery of angiogenic growth factors to achieve synergy between the two approaches for the treatment of ischemically damaged tissues (Ikenaga et al., 2001; Nabel, 2002). Interplay between anti- and pro-angiogenic growth factors is critical for the cascade of reactions involved in blood vessel formation and maturation. Indeed, the angiogenic process depends on the sequential and cooperative effects of various growth factors in order to form functional vessels (Gale and Yancopoulos, 1999).

1.2.1 Angiogenesis involves angiogenic growth factors and other stimuli

The pivotal role of vascular endothelial growth factor (VEGF) as a potent inducer of angiogenesis has been well studied (Ferrara and Henzel, 1989; Ferrara et al., 1991; Isner, 1998; Carmeliet and Collen, 1999b; Dvorak et al., 1999; Eriksson and Alitalo, 1999; Ferrara, 1999; Neufeld et al., 1999). It has direct and specific mitogenic effects on endothelial cells (Ferrara and Henzel, 1989). Unlike the other heparin-binding angiogenic growth factors, VEGF possesses a typical secretory signal sequence on the amino terminus which promotes its release from the intact cells (Leung et al., 1989). The gene for human VEGF is organized into eight exons and as a result of alternative splicing, at least six transcripts encoding mature monomeric VEGF containing 121, 145, 165, 183, 189 and 206 amino acid residues ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$) have been reported to date (Robinson and Stringer, 2001). $VEGF_{165}$ binds to two high-affinity receptors on the surface of endothelial cells, designated as *fms*-like tyrosine kinase-1 (Flt-1/VEGF-R1) (Shibuya et al., 1990) and kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1/VEGF-R2) (Terman et al., 1992; Carmeliet, 1999a). KDR/Flk-1/VEGF-R2 is the principle receptor for $VEGF_{165}$ and is expressed by both endothelial cell progenitors as well as hematopoietic stem cells. $VEGF_{165}$ triggers the angiogenesis cascade by binding to this receptor during vasculogenesis in embryonic development to induce the differentiation, proliferation and migration of endothelial cells to form the initial network of primitive tubules (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999a, b).

Besides VEGF, basic fibroblast growth factor (bFGF) initiates the angiogenesis cascade upstream of VEGF by inducing endothelial cells to produce urokinase-type plasminogen activator which modifies the extracellular matrix to allow subsequent invasion of the activated capillary endothelial cells (Montesano et al., 1986). With promising data from animal studies, bFGF has been employed in clinical trials (Lazarous et al., 2000; Lederman et al., 2002).

The role of Ang-1 is crucial for further remodeling and maturation of the initially immature vasculature. Ang-1 exists predominantly as a homo-multimer, held together by coiled-coil domains and disulfide cross-links and signals through a receptor-like tyrosine kinase Tie2/Tek. These receptors are expressed specifically on endothelial cells and early hematopoietic cells (Iwama et al., 1993; Sato et al., 1993; Korhonen et al., 1994; Sato et al., 1995; Davis et al., 1996; Procopio et al., 1999; Huang et al., 2000). Mesenchymal cells first express Ang-1 that in turn activates the Tie2 receptor on the endothelial cells to produce and release a recruiting signal. This recruiting signal would in turn induce the differentiation of the mesenchymal cells into peri-endothelial support cells such as pericytes and smooth muscle cells (Rohovsky et al., 1996). Interaction between the endothelial cells and the surrounding support cells to form a mature vessel wall will then solidify and stabilize the newly formed blood vessel (Davis et al., 1996; Suri et al., 1996). Following vessel maturation, Ang-1 continues to play an important role in maintaining the stability of the mature vasculature. In the absence of maturation factors such as Ang-1, VEGF stimulates vascular permeability in the small immature blood vessels, composed merely of the endothelial cell lining. The increased permeability causes leakage of plasma proteins, with resultant hemangioma formation. *In vitro* experiments have also

shown that Ang-1 potently induces chemotactic response (Witzenbichler et al., 1998), network formation (Papapetropoulos et al., 1999), sprouting (Koblizek et al., 1998; Kim et al., 2000a) and survival in apoptosis (Papapetropoulos et al., 1999; Kim et al., 2000b) but has no mitotic effect (Davis et al., 1996; Kim et al., 1999). Hence, although Ang-1 is incapable of mediating endothelial cell growth or tubule formation as VEGF, it seems to play a role in later stages of vessel development.

Another important member of the angiopoietin family is angiopoietin-2 (Ang-2), which blocks the constitutive stabilizing action of Ang-1 (Maisonpierre et al., 1997; Goede et al., 1998). This occurs when Ang-2 competes with Ang-1 for the Tie2 receptor resulting in the inhibition of the Ang-1/Tie2 signal. Such an event results in a loosening of the tight vascular structure that has been maintained previously by the Ang-1/Tie2 signal. Thus, this leads to the opening up of the vessel structure to allow protease degradation of the basement membrane surrounding the endothelium. The exposed endothelial cells are now easily accessed by angiogenesis inducers such as VEGF. If VEGF is indeed present, the endothelial cells become activated to migrate and proliferate, thereby producing new capillary sprouts and in turn tubes in a process termed 'angiogenic sprouting'. Such sprouting event is followed by a shift in the local balance of Ang-1/Ang-2 back in favor of Ang-1 to allow maturation and stabilization of the newly formed vessel.

In addition to the above-mentioned factors, many other proangiogenic factors influence the angiogenic process, amongst which are members of the transforming growth-factor β-family, epidermal growth factor, tumor necrosis factor-α, platelet-derived growth factor BB, hepatocyte growth factor, interleukin-1a and nitric oxide, whose roles include stimulating VEGF expression as well as stabilization of newly

formed blood vessels. (Murohara et al., 1998; Hellstrom et al., 1999; Hirschi et al., 1999; Carmeliet, 2000a; Onimaru et al., 2002). Furthermore, a myriad of other gene products, ranging from transcription factors to members of the Notch family, also play crucial role in vessel formation (Carmeliet, 2000a).

On top of these, angiogenesis is also strongly regulated by hypoxia (Semenza, 1999) and metabolic stimuli such as hypoglycemia and acidosis. There is evidence that hypoxia upregulates transcription of VEGF mRNA and the vessel stability is enhanced in terms of increased vascular smooth muscle cells due to stimulation by the hypoxia-inducible factor-1 (HIF-1). In rats chronically exposed to hypoxic condition, the mRNA for both VEGF and KDR/Flk-1/VEGF-R2 is upregulated in the lung tissue (Tuder et al., 1995). Hence, hypoxia is a major driving force for angiogenesis (Plate et al., 1992; Shweiki et al., 1992).

1.2.2 Delivery strategies for therapeutic angiogenesis

For therapeutic angiogenesis, angiogenic growth factors have been administered either as recombinant proteins or by way of gene transfer (Table-1).

Table-1: VEGF protein/gene transfer in animal model of limb ischemia

Delivery technique	Animal model	Reference
Recombinant protein	Rabbit	Bauters et al., 1994, 1995
	Rabbit	Takeshita et al., 1994a, b, 1995
	Rabbit	Hopkins et al., 1998
	Rat	Chang et al., 2003
'Naked' plasmid DNA	Rabbit/Rat	Takeshita et al., 1996/1998
	Rabbit	Chae et al., 2000
	Rabbit	Shyu et al., 2003a
Adenoviral vector	Rabbit	Hershey et al., 2003
Adeno-associated virus	Rat	Arsic et al., 2003
Liposome	Rabbit	Gowdak et al., 2000a
	Rat	Neumeister et al., 2001

However, because of limited availability of human-quality grade recombinant protein and the cost effectiveness of the procedure, the feasibility of using recombinant angiogenic growth factor proteins is currently limited. On the contrary though, gene transfer strategy makes use of the gene encoding for the growth factor to be administered as a template for production of the therapeutic protein. Such therapeutic strategy harnesses the patient's own cellular machinery to produce the growth factors rather than relying on pharmacotherapy. The cells transduced with the exogenous gene serve as reservoirs for the growth factor protein for a certain period of time (Leiden, 2000) and as

such avoids repeated dose administration (Takeshita et al., 1994a). The angiogenic gene can be delivered as a 'naked' plasmid DNA or carried in a vector cassette (Takeshita et al., 1996; Tsurumi et al., 1996). The delivery has been achieved via a variety of approaches, such as direct injection and electroporation (Takeshita et al., 1996; Tsurumi et al., 1997; Takeshita et al., 1998). Intravenous administration of 'naked' plasmid DNA does not elicit an autoimmune response (Nabel et al., 1992) as double stranded DNA is minimally antigenic because the bases are in essence 'shielded' from immunologic surveillance by the double helices (Schwartz and Stollar, 1985). The delivery of 'naked' plasmid DNA however, suffers the drawback of low transfection efficiency. This is most likely connected to the large molecular size of the plasmid (13-15 kb) which account for the poor uptake by cells. To overcome the problem of transfection efficiency, vector-mediated gene transfer using viral as well as non-viral vectors, have thus been designed to effect therapeutic angiogenesis (Roessler et al., 1994; Schwartz et al., 1995; Goldman et al., 1997). The viral vectors including replication-deficient adenovirus, retrovirus, lentivirus, and adeno-associated virus, are often efficiently taken up by the host cells via receptor-mediated mechanisms or endocytosis upon their delivery to the target tissue (Table-2). Of these, adenoviruses (Ad) have been widely used for gene therapy, due to their ability to efficiently transduce non-dividing cells (Gilgenkrantz et al., 1995; Muhlhauser et al., 1996; Crystal, 1997). Moreover, adenoviral vectors may be amplified and produced to high titres (10^{11} – 10^{13} virus particle ml^{-1}) besides being capable of infecting post-mitotic cells and accommodating transgene cassette of up to 10 kb. They are made replication deficient by producing point mutations, deletions, insertions and combinations directed toward a specific adenovirus gene(s), such as the early region 1

(E1) gene. Unfortunately, the use of viral vector-based gene delivery has remained questionable due to its serious immunologic concerns. Adverse reactions from incomplete inactivation of the viral replication machinery lead to life-threatening systemic inflammatory-like reactions (Leiden, 2000; Verma, 2000). In addition, administration of adenoviral vectors immediately after induction of acute ischemia in rat hind limbs cause inflammation and subsequent limb gangrene that seemed to be aggravated by the adenovirus capsid protein (Brevetti et al., 2001). Pioneering work of Felgner et al. (1987) introduced lipofection for non-viral gene transfer. The winning characteristics of lipofection are its low cytotoxicity, low immunogenicity and non-integration into host genome. Once again though, lipofection suffers the drawback of low transfection efficiency, hence its use compromises between safety and gene delivery efficiency.

Table-2: Advantages and disadvantages of viral and non-viral vector delivery systems

Delivery System	Type	Advantages	Disadvantages
Viral	Adenovirus, adeno-associated, retrovirus, herpes simplex	High transfection efficiency	Reduction in the size and structure of gene to be inserted, triggers immune response, lacks target specificity, problems in safety, toxicity and large scale production, chances of permanent integration into the host cell genome, risk of tumorigenesis
Non-viral vectors	Cationic lipids, liposomes, emulsion, polymer, nano-particles	Easier manipulation, flexibility in DNA insertion size, site-specific targeting, safety, large-scale production, non-immunogenic, non-pathogenic	Lower transfection efficiency, transient gene expression, instability in serum containing environment

1.2.3 Therapeutic angiogenesis using VEGF

Despite numerous investigations, the challenge remains in identifying the factor or combination of factors that will stimulate functional neovascularization. VEGF was previously perceived to fit this criterion. Various animal studies have shown augmentation of the collateral blood flow and function in hind limb ischemia models (Takeshita et al., 1994a, b; Walder et al., 1996; Baffour et al., 2000; Cherng et al., 2000). However, recent reports on the use of VEGF for therapeutic angiogenesis in PVD treatment have revealed untoward effects of the therapy (Baumgartner, 2000; Masaki et al., 2002). Overexpression of VEGF results in the formation of non-functional leaky vessels in experimental animals, while plasmid-based intramuscular VEGF gene transfer has led to transient edema in human subjects (Baumgartner et al., 1998; Schainfeld et al., 1999; Thurston et al., 1999, 2000). Other undesirable effects of VEGF include formation of "angioma-like" fragile capillary network without connections with the pre-existing vessels (Springer et al., 1998; Carmeliet, 2000a; Lee et al., 2000). This was further confirmed by Masaki et al. (2002) who showed that VEGF gene transfer to limbs led to an accelerated amputation associated with massive muscular edema, necrosis and disturbed regeneration. The study also observed no significant increase in blood perfusion though increased number of capillaries was apparent. This latter notion was consistent with a report from Hershey and colleagues (2003) who demonstrated that VEGF gene transfer to limbs led to a robust increase in capillary density. However, there was no significant improvement in the collateral blood flow. Taken together, these findings showed that VEGF overexpression stimulated sprouting of immature leaky blood conduits that lack encasement with vascular smooth muscle cells. In a follow-up study,

Ozawa et al. (2004) demonstrated a discrete threshold level in VEGF dosage (~70ng/10^6 cells/day), below which normal, non-leaky, stable capillaries are induced and above which hemangioma growth occurs. The study used a clonal population of myoblasts (muscle precursor cells) carrying a retrovirally transduced VEGF gene. This homogenous population of genetically modified myoblasts allows constitutively long-term VEGF gene expression at precisely controlled low dosage. Under these conditions, VEGF remarkably promoted growth of stable, non-leaky capillaries of uniform size independent of continued VEGF expression. Thus, long-term continuous delivery of VEGF, when maintained below a threshold microenvironmental level, can lead to normal angiogenesis without other exogenous growth factors. On the other hand, overexpression of VEGF above this threshold level, without the accompaniment of maturation factors such as Ang-1, leads to aberrant angiogenesis. This showed that uncontrolled and imbalance of angiogenic signals may have deleterious effects and may not always contribute to the formation of functional neovascularization.

1.2.4 Therapeutic angiogenesis using FGF

Besides VEGF, members of the FGF family have also been widely used for therapeutic angiogenesis. In a study by Baffour et al. (1992), bFGF was administered intramuscularly to rabbits with acute hind limb ischemia. Upon completion of treatment at 14 d, angiography and necropsy measurement of capillary density showed evidence of augmented collateral vessel formation in the lower limb compared with controls. It has also been reported that bFGF accelerates wound healing in several animal models (McGee et al., 1988; Mooney et al., 1990). Surprisingly though, a comparison between

Ad-FGF-4 and Ad-VEGF by Rissanen et al. (2003), revealed that intramuscular injection of both these vectors resulted not only an increased in collateral growth, popliteal blood flow and muscle perfusion, but also demonstrated the side effect of vascular permeability and edema in transduced muscles of rabbit hind limb ischemia. FGF-4 is capable of inducing vascular permeability, therapeutic angiogenesis and arteriogenesis comparable to that of VEGF. The underlying reason to this similarity may be related to FGF-4 mediated upregulation of endogenous VEGF level, which may in part explains the effects thought to be VEGF specific.

1.2.5 Therapeutic angiogenesis using nitric oxide synthase

Previously thought of as a noxious gas, nitric oxide (NO) is now recognized as an important mediator of vascular responsiveness. It is synthesized in the vascular endothelium utilizing the enzyme nitric oxide synthase (NOS) and diffuses in the adjacent vascular media, where it has a vasodilatory action (Palmer et al., 1987).

The role of nitric oxide in vasodilatation makes it an attractive candidate for angiogenic therapy. This is supported in a recent study by Brevetti et al. (2003) who demonstrated that adenoviral overexpression of NOS in a rat model of hind limb ischemia increased blood flow and tissue oxygenation to the limb without a significant change in capillary density.

1.2.6 The approach to achieve angiogenic synergy

Considering that angiogenesis involves interplay between various growth factors and effector cells, which is very delicately poised to favor the formation of new blood vessels, simultaneous delivery of multiple angiogenic factors may lead to better prognosis. Indeed, the approach of using a combination of factors to achieve angiogenic synergy has shown more promise for the treatment of PVD. Asahara and colleagues (1995) investigated the simultaneous delivery of VEGF and FGF to achieve angiogenic synergy. In a rabbit model of hind limb ischemia, significantly greater capillary density was achieved in experimental animals receiving both VEGF and bFGF as compared to the ones receiving VEGF or bFGF alone. Furthermore, there was progressive increase in the luminal diameter in the experimental group. This was confirmed in a similar study by Kondoh et al. (2004) who reported that combined gene delivery of VEGF and bFGF produced additive or synergistic effects of collateral development as compared to those receiving either gene alone. Cao et al. (2003) combined FGF-2 and platelet-derived growth factor (PDGF)-BB to synergistically induce vascular networks. The results revealed that simultaneous administration of both factors stimulated functionally stable arteriogenesis with significantly improved blood flow.

A comparative study between VEGF and FGF-2 gene therapy revealed that FGF-2 gene therapy alone was effective to treat hind limb ischemia with no deleterious effects (Masaki et al., 2002). Apparently, the underlying mechanism of repair was the concerted action of FGF-2 and VEGF after FGF-2 gene administration which strongly evoked endogenous VEGF expression. The duo combination of these factors led to functionally mature neovascularization with a positive smooth muscle actin coating. More

interestingly, FGF-2 acts as a muscle regenerating factor and hence, FGF-2 gene transfer not only induced the angiogenesis cascade but also induced muscle regeneration (Lefaucheur et al., 1995; Lefaucheur et al., 1996).

In another study using systemic delivery of Ang-1 gene by adenoviral vector, angiogenic synergy between VEGF and Ang-1 reduced vascular leakage induced by mustard oil and VEGF in adult vessels (Thurston et al., 2000). Thus, it appears that VEGF administration, together with Ang-1 seemingly stabilizes the neovasculature. More importantly, combining transgenic Ang-1 and VEGF had an additive effect on neovascularization and produced the leakage-resistant vessels typical of Ang-1 overexpression (Thurston et al., 1999; Arsic et al., 2003; Shyu et al., 2003a). These findings were further confirmed by Chae et al. (2000) who combined VEGF and Ang-1 gene delivery to rabbit ischemic hind limb for enhanced effect on blood flow and capillary formation. Thus, combined growth factor administration seems to be an important requisite in therapeutic terms and this raises the suitability of VEGF and Ang-1 synergism for therapeutic angiogenesis.

1.2.7 VEGF and Ang-1 synergy: an ideal combination for therapeutic angiogenesis

Despite achieving significant improvements in collateral development through administration of various angiogenic growth factors and other stimuli in experimental animal models, it is still premature to gauge if the exciting results obtained in animals can be recapitulated in humans. No doubt the 'proof of concept' studies in these animal models (Table-4) have revealed the safety and feasibility of therapeutic angiogenesis for the treatment of PVD and have now formed the basis of various human clinical trials (Table-3). Indeed, VEGF (Isner et al., 1996a, b; Baumgartner et al., 1998; Makinen et al., 2002; Shyu et al., 2003b) and bFGF (Lazarous et al., 2000; Cooper et al., 2001; Lederman et al., 2002) have gone one step further by being used in Phase I and II clinical trials as well. However, results have been rather conflicting with respect to the demonstration of a salutary biologic effect. Clearly, further studies are needed to elucidate the mechanism involved in successful therapeutic angiogenesis.

Table-3: Growth factor used for therapeutic angiogenesis in clinical phase I and II studies of lower limb ischemia

Growth factor	Reference
VEGF	Isner et al., 1996a, b; Baumgartner et al., 1998; Makinen et al., 2002; Shyu et al., 2003b.
bFGF	Lazarous et al., 2000; Cooper et al., 2001; Lederman et al., 2002

Table-4: Growth factor(s) used for therapeutic angiogenesis in animal models of limb ischemia

Growth factor	Reference
VEGF	Bauters et al., 1994, 1995; Takeshita et al., 1994a, b, 1995, 1996, 1998; Walder et al., 1996; Hopkins et al., 1998; Cherng et al., 2000; Gowdak et al., 2000a, b; Lu et al., 2001; Neumeister et al., 2001; Smythe et al., 2002; Chang et al., 2003; Hershey et al., 2003.
VEGF and Ang-1	Chae et al., 2000; Arsic et al., 2003; Shyu et al., 2003a
VEGF and bFGF	Asahara et al., 1995; Kondoh et al., 2004
bFGF	Baffour et al., 1992; Chleboun et al., 1992; Chleboun and Martins, 1994; Yang et al., 1996, 2000
aFGF	Tabata et al., 1997
FGF-2	Masaki et al., 2002
FGF- 4	Rissanen et al., 2003
PDGF-BB & FGF-2	Cao et al., 2003
ECGF	Pu et al., 1993
PAR-2AP	Milia et al., 2002

PDGF= Platelet-derived growth factor
ECGF= Endothelial cell growth factor
PAR-2AP= Protease activated receptor-2 activating peptide

Having taken into consideration these previous studies, we believe that synergistic interaction between VEGF and Ang-1 is the right combination for effective therapeutic vascularization in the treatment of limb ischemia. This is partially because they contribute to a combined beneficial effect of what the other angiogenic growth factors and stimuli have to offer. For instance, VEGF can also stimulate vasodilatation

(Takeshita et al., 1998) by acting upstream of the NO pathway. Apparently, VEGF binds to its VEGF-R2 receptor found on endothelial cells to stimulate production of NOS that is in turn responsible for synthesis of NO (Kroll and Waltenberger, 1998). The direct use of NO for treatment of ischemic limb can improve blood flow through vasodilatation (Brevetti et al., 2003), however one must be cautious of the other effects that NO has on other systems of the body. Nitric oxide also inhibits the aggregation of platelets and thus keeps inappropriate clotting from interfering with blood flow. Overexpression of NO may instead cause unwanted continuous bleeding. Moreover, some motor neurons of the parasympathetic branch of the autonomic nervous system release NO as their neurotransmitter and their overexpression may thus produce unwanted continuous activation of a particular neuronal pathway. Using VEGF, on the other hand, provides a safer option as its main role is limited to the vascular system. The effects of VEGF-mediated synthesis of NO would be less direct and thus most probably limited to vasodilatation.

Besides, VEGF overexpression in the accompaniment of a maturation factor like Ang-1, plays a major role in formation of functional new blood vessels. VEGF plays the pivotal role by inducing the differentiation, proliferation and migration of endothelial cells to form the initial network of primitive tubules. Ang-1 influences the remodeling and maturation of this initially immature vasculature by recruiting support cells such as pericytes and smooth muscle cells to form mature and stable newly formed blood vessels. Following vessel maturation, Ang-1 continues to play an important role in maintaining the stability of the mature vasculature. In the absence of maturation factor, VEGF stimulates vascular permeability in the small immature blood vessels that are merely

blood vessel conduits. The increased permeability causes leakage of plasma proteins, with resultant hemangioma formation, thereby emphasizing the need for both growth factors.

Whilst FGF has also been reported to activate the angiogenic cascade by stimulating VEGF expression, VEGF may still be the preferred choice for treatment of ischemic limb. FGF, unlike VEGF, lacks a secretory signal sequence and is therefore not actively secreted from cells following gene transfer (Tabata et al., 1997). It is therefore a necessity that FGF gene be modified by adding a signal sequence prior to transfection in order to achieve a clinical response. This added difficulty has caused less interest in developing gene transfer methods for FGF compared with VEGF. In addition, though FGF is a powerful mitogen of the endothelial cells, it is not endothelial cell specific like the other angiogenic growth factors such as VEGF and Ang-1. This choice of VEGF over FGF is further supported in a comparative study between VEGF and FGF-2 by Palmer-Kazen et al. (2004). The outcome of the study indicated that there are elevated concentrations of FGF-2 in calf muscle, whereas VEGF concentrations do not appear to be higher in the ischemic part of the leg in patients with critical limb ischemia. These findings suggest that VEGF supplementation may be a more appropriate strategy for therapeutic angiogenesis to the calf area for critical limb ischemia than FGF-2.

1.3 Cell transplantation for angiogenesis

Despite encouraging results from direct protein or gene transfer of angiogenic growth factors, the approach is still fraught with problems including neoplasms and plaque angiogenesis. With the view to overcome these problems, the focus is now

shifting to combo-therapy involving cell transplantation together with therapeutic angiogenesis (Hamano et al., 2001; Ikenaga et al., 2001). The angiogenic effect induced by implantation of of various cells with intrinsic ability to express various growth factors or genetically modulated to express angiogenic factors have recently been examined. Bone marrow cell implantation is a safe and effective choice for the treatment of ischemic limb disease (Hirakata et al., 2003). Al-Khaldi and colleagues (2003) showed that autologous marrow stromal cells implantation induced a neovascular response associated with significant increase in blood flow to rat ischemic limbs. Similar results have been documented by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs of athymic nude rats to induce collateral vessels (Iba et al., 2002).

1.4 *Ex vivo* gene therapy for therapeutic angiogenesis

The approach of combo-therapy for therapeutic angiogenesis is gaining popularity and involves removing cells from the body to genetically modulate them with the angiogenic growth factor genes in culture before introducing them back into the host (Suzuki et al., 2001; Iwaguro et al., 2002; Haider et al., 2004). The genetically modulated cells become integrated into the host tissue to provide a sustained and localized elevation in the growth factor level. The duration of gene expression is determined by the vector which has been used for gene transfer into the cells. For instance, whilst retroviral vectors allow stable integration of the exogenous genes into the host cell genome, adenoviral vectors merely remain outside of the host genome as an episome. As such, retroviral-mediated gene transfer allows long-term stable expression of the angiogenic growth

factors wherelse its adenoviral counterpart allows only transient expression of the factor. The genetically altered cells can be fully characterized *in vitro* before transplantation to ensure expression of the recombinant products. Moreover, the isolated cells generally avoid inadvertent transduction of cells other than the target cells. By contrast, direct intramuscular 'naked' plasmid DNA injection, viral-mediated gene delivery or other vector-mediated gene delivery are often limited by the variable transduction efficiency, thus making secretion levels difficult to predict. Furthermore, problematic immunological effects associated with most other methods can be avoided with *ex vivo* approaches by transplantation of autologous cells. The choice of cell suitable as a vector for delivery of the growth factor gene however, remains a debatable issue. Skeletal myoblast appears to be most suitable due to their superior characteristics (Table-5).

Table-5: Advantages of skeletal myoblasts for cell transplantation

- Established protocols for the isolation and expansion of primary skeletal myoblasts from animal and human skeletal muscles.
- High growth potential *in vitro* and easy maintenance in culture over a longer period of time by passaging.
- Easy to genetically manipulate *in vitro* and extensively characterized before re-implantation back into muscle.
- Ability to fuse with pre-existing muscle fibers upon intramuscular transplantation.
- Strong resistance to ischemia *in vivo*.
- No tumorigenic and toxicity issues after transplantation.
- The availability from autologous source avoids associated ethical and donor availability issues.

1.4.1 Skeletal myoblasts participate in myogenesis

During myogenesis, mononucleated muscle precursor cells called myoblasts, fuse with one another. The fusion process is preceded by a complex series of sequential events including cell alignment, adhesion and intercellular communication. The fusion event itself is rather peculiar as it does not involve breakdown of nuclear membrane. Rather, the nuclei of the fusing cells remain as independent discrete units and perform their function independently. They form multinucleated myotubes that share a common pool of genetic material. Each myotube contains centrally located nuclei which are arranged in linear order. Upon establishing the neuronal and vascular connections, these myotubes undergo further differentiation into myofibrils and thence into the mature fully functional muscle fibers. In the event that these myotubes fail to develop any such connections, they simply degenerate.

As these muscle fibers are terminally differentiated tissues, they are no longer capable of undergoing mitosis for maintenance purpose. Rather, muscle fibers are maintained by a unique resident population of mononuclear myogenic precursors called the satellite cells. These cells, which take residence between the sarcolemma and the basal lamina of the muscle fibers, account for only approximately 5 % of the total nuclei present within muscle fibers (Bischoff, 1994). They divide at a slow rate to sustain both self-renewal and growth of differentiated tissue. However, in response to muscle injury or in individuals inflicted with chronic degenerative myopathies, satellite cells migrate to sites of muscle fiber damage and divide extensively in order to produce sufficient mononuclear myoblasts that later fuse with each other and also with the ends of damaged myofibers to repair and repopulate the traumatized muscle. Unfortunately, the self-

renewal potential of adult satellite cells is limited, decreases with age and can be exhausted by a chronic regenerative process such as that characteristic of severe muscular dystrophies.

Besides satellite cells, recent studies have suggested that stem cells from sources other than muscle, could contribute to muscle regeneration. Indeed, this was supported by evidence that progenitor cells isolated from bone marrow (Ferrari et al., 1998; Gussoni et al., 1999), the embryonic vasculature (De Angelis et al., 1999), the neuronal compartment (Clarke et al., 2000; Galli et al., 2000), and various mesenchymal tissues (Young et al., 2001 a, b) can also differentiate into the myogenic lineage.

1.4.2 Skeletal myoblasts as carriers of exogenous genes

Beyond their role in myogenesis and muscle repair, normal skeletal myoblasts have been isolated, cultured and transplanted *in vivo* to treat a variety of diseases. Although the number of satellite cells in adult skeletal muscle is low (only about 5 %), protocols have been established for the isolation and expansion of primary skeletal myoblasts from both animal and human skeletal muscles (Rando et al., 1994). Hence, it is technically feasible to isolate and propagate sufficient numbers of skeletal myoblasts to augment muscle function *in vivo*. The role of skeletal myoblasts as carriers of exogenous genes has been extensively studied in animal models (Table-6).

Table-6: Studies on myoblast-mediated gene therapy.

Gene delivered	Cell source	Animal model	Reference
HGH	PMM	Mouse	Barr et al., 1991; Dhawan et al., 1991
CF-IX	PMM	Mouse	Dai et al., 1992
	C2C12	Mouse	Yao et al., 1992
	PMM	Mouse	Yao et al., 1994
Erythropoietin	C2C12	Mouse	Hamamori et al., 1994,1995
	PMM	Mouse	Bohl et al., 1997

C2C12= A myogenic cell line; CF= Coagulation factor; HGH= Human growth hormone
PMM= Primary mouse myoblast

Springer et al. (1998) have made use of the duo myoblast and VEGF gene combination for transplantation into the non-ischemic muscles of immunodeficient SCID mice. However, due to retroviral transduction of myoblasts, there was long-term stable expression of the angiogenic growth factor. Consequently, the constitutive overexpression of VEGF after implantation led to an increased in vascular structures and hemangioma formation. The deleterious effect of retroviral transduction is also highlighted in a recent report that showed retrovirally transduced human cells have the potential of causing malignant transformation (Hacein-Bey-Abina et al., 2003). To overcome this problem, a tetracycline-inducible system has been developed that permits interruption of the expression of the gene after a given period or to switch it on at a desired time point (Kringstein et al., 1998; Blau and Rossi, 1999). The ability to control the expression of the VEGF gene by using regulatable promoters is indeed a significant development. The differential expression of the gene alone gave rise to very different vessel phenotypes as reported in a recent study by Springer et al. (2003). Apparently, in

areas adjacent to the implantation site of the VEGF-producing myoblasts, the typical pattern of the capillary network in skeletal muscle was replaced by numerous smooth-muscle-cell-coated vessels. Similar phenotypes were also observed in areas of reduced myoblast engraftment. Lu et al. (2001) delivered recombinant VEGF to mouse ischemic hind limbs using implantable bioartificial muscle tissues made from retrovirally transduced VEGF-expressing mouse primary skeletal myoblasts. The outcome was a 2- to 3-fold increase in capillary density with no evidence of hemangioma formation. Based on these findings and encouraging experimental evidence, angiomyogenesis using myoblasts transduced with angiogenic growth factor genes offers an attractive potential option for treatment of PVD.

1.4.3 Pitfalls in myoblast transplantation for gene delivery

One of the basic requirements of any cell-based therapy is that it requires an effective dispersion of the cells from the site of injection. In the case of myoblast transplantation, various strategies have been adopted to promote the migration of donor myoblasts through the interstitial connective tissue into host muscle. In order to facilitate such migration, enzymes have been used to degrade the extracellular matrix in the host muscle (Ito et al., 1998; Caron et al., 1999; El Fahime et al., 2000; Torrente et al., 2000). Alternatively, multiple injection site delivery approach has been adopted to facilitate donor cell dispersal. However, the damaging effect of these methods on the host tissue may limit their clinical relevance. More attractive and clinically viable alternatives include enhancement of their proliferation and delaying their fusion through genetic modulation or the administration of exogenous myoblasts mitogens. Skeletal myoblasts

grown with 20 μg/mL of Concanavalin-A two days before transplantation into mice skeletal muscles showed increased migration from the injection site (Ito et al., 1998). Similarly, absence of the myogenic regulatory gene MyoD in donor skeletal myoblasts significantly enhances their migration into host muscle from sliced muscle graft, however, with as yet unknown mechanism (Smythe and Grounds, 2001a).

Another problem associated with myoblast transplantation is the onset of rapid and massive death of grafted cells observed after the transplantation (Beauchamp et al., 1994; Huard et al., 1994a). Besides other factors, the host immune response has been implicated in the rapid and massive death of the donor cells (Smythe et al., 2000b). The use of immunosuppression has led to the long-term survival of grafts of muscle and myoblasts (Kinoshita et al., 1994, 1996a; Lochmuller et al., 1996). More interesting though, are studies that reported a significantly higher number and better persistence of dystrophin-positive myofibers demonstrated when the injected muscle and donor myoblasts expressed a similar myosin heavy chain (MyHC) in comparison with myoblasts transfer between host muscle and donor myoblasts that were not matched for MyHC (Qu et al., 2000). These results suggest that careful matching between the injected myoblasts and injected muscle for the MyHC expression can improved the efficiency of myoblasts-mediated transfer to skeletal muscle. There is compelling evidence that skeletal myoblasts grown with 100 ng/mL bFGF exhibit reduced myotube formation *in vitro* and higher level of survival *in vivo* as compared to those cultured without bFGF (Kinoshita et al., 1995, 1996b). Morevoer, the problem of massive cell death is currently being compensated by injection of a larger number of donor cells.

In summary, combo-therapy involving skeletal myoblasts and therapeutic angiogenesis offers a promising avenue for treatment of ischemic limb disease. *Ex vivo* angiogenic growth factor gene delivery by myoblast-mediated transfer offers a potential therapeutic option for limb ischemia. The outcome of the procedure may be improvised through angiogenic synergy by using a bicistronic vector.

1.5 Statement of problem and hypotheses

1.5.1 Project aim

The idea of combining myogenesis and angiogenic synergy through the use of a combination of angiogenic factors is interesting. As mentioned previously, single growth factor gene delivery into ischemic animal models is insufficient for therapeutic purposes. In fact, there is clear evidence now, as shown in studies of animals and human patients, that delivery of a single angiogenic agent can cause serious complications (Carmeliet, 2000b; Lee et al., 2000; Celletti et al., 2001; Epstein et al., 2001). Indeed, the development of a functional vascular system requires more than a single growth factor, their receptors and intracellular signals (Folkman and D'Amore, 1996; Risau, 1997; Carmeliet, 2000a).

The use of skeletal myoblasts as carriers of various exogenous genes has been widely studied in experimental animal models (Barr and Leiden, 1991; Dhawan et al., 1991; Dai et al., 1992; Yao and Kurachi, 1992; Yao et al., 1994; Hamamori et al., 1994,1995; Bohl et al., 1997). However, its use as a vehicle for angiogenic genes such as VEGF is currently limited to studies focusing on experimental heart failure models (Taylor et al., 1998; Scorsin et al., 2000; Suzuki et al., 2000) but not that of ischemically

damaged hind limbs. It was therefore the primary aim of the present research to investigate the feasibility and efficiency of transplanting autologous skeletal myoblasts transiently expressing $VEGF_{165}$ and Ang-1 genes to treat hind limb ischemia.

1.5.2 The bicistronic adenoviral vector construct

A novel adenoviral bicistronic vector encoding for the human $VEGF_{165}$ and human Ang-1 genes under the control of the same promoter was used to transduce these myoblasts. This vector was constructed in Dr Ge Ruowen, Angiogenesis Laboratory, National University of Singapore. A plasmid-based system approach similar to that reported by Bett et al. (1994) was used to insert the foreign genes $VEGF_{165}$ and Ang-1 into the Ad vector. Three different plasmids - pIRES, pMH5 and pJM17 - were involved in this system. pIRES was used because it is a eukaryotic bicistronic expression plasmid which carries an internal ribosome entry site (IRES) sequence [from the *Encephalomyocarditis virus* (EMCV)] flanked by two multiple cloning sites (MCS A and MCS B) in the expression cassette. Cloning of the $VEGF_{165}$ and Ang-1 genes into each of these MCS will generate the combinatorial 5'-$VEGF_{165}$+IRES+Ang-1-3' sequence. Such a gene arrangement is essential since the IRES sequence sandwiched between these two foreign genes allows for the translation of the two genes from the same mRNA transcript. In its absence, translation of the downstream cistron of the bicistronic transcript is extremely inefficient as only a small proportion of ribosomes recommence scanning after completing translation of the upstream cistron and are capable of initiating downstream cistron translation (Kozak, 1989; Jackson et al., 1990). Thus, insertion of an IRES

element between the two ORFs effectively promotes translation of the downstream cistron in a manner which is independent of the 5'-end of the RNA.

The next is to clone the 5'-$VEGF_{165}$+IRES+Ang-1-3' sequence directly into an Ad vector using the conventional *in vitro* ligation process. Unfortunately, the large size of the Ad genome limits the number of unique, useful restriction sites available for *in vitro* manipulation. Hence, it is easier to first clone the 5'-$VEGF_{165}$+IRES+Ang1-3' gene sequence into the adenoviral shuttle plasmid, pMH5. pMH5 contains Ad type 5 sequences from bp 22 to 5790 with a deletion of E1 sequences from bp 342 to 3523. At the position of deletion, a 1.4 kb fragment of the murine cytomegalovirus (MCMV) immediate early gene 1 enhancer/promoter is inserted. The inserted promoter is followed by a polycloning oligonucleotide with multiple restriction enzyme sites, as well as the simian virus 40 (SV40) polyadenylation signal. Cloning of the 5'-$VEGF_{165}$+IRES+Ang1-3' transgene within this polycloning oligonucleotide would thus allow the insert to be flanked by MCMV promoter and SV 40 polyadenylation signal as well as Ad E1 sequences. The resultant shuttle plasmid is co-transfected with pJM17 (a plasmid containing almost the entire Ad type 5 genome) into 293 cells, *in vivo* homologous recombination within a sequence common to both plasmids (that is E1 of Ad sequence in this case) will target the 5'-$VEGF_{165}$+IRES+Ang1-3' gene sequence to the desired position in the pJM17 vector. The end product is an E1-defective recombinant adenovirus that is replication-incompetent, carrying the $VEGF_{165}$ and Ang-1 genes (Figure 1; adopted from Niagara Muhammad Idris, NUS Bachelor of Applied Science Honours Thesis, 2001/2002).

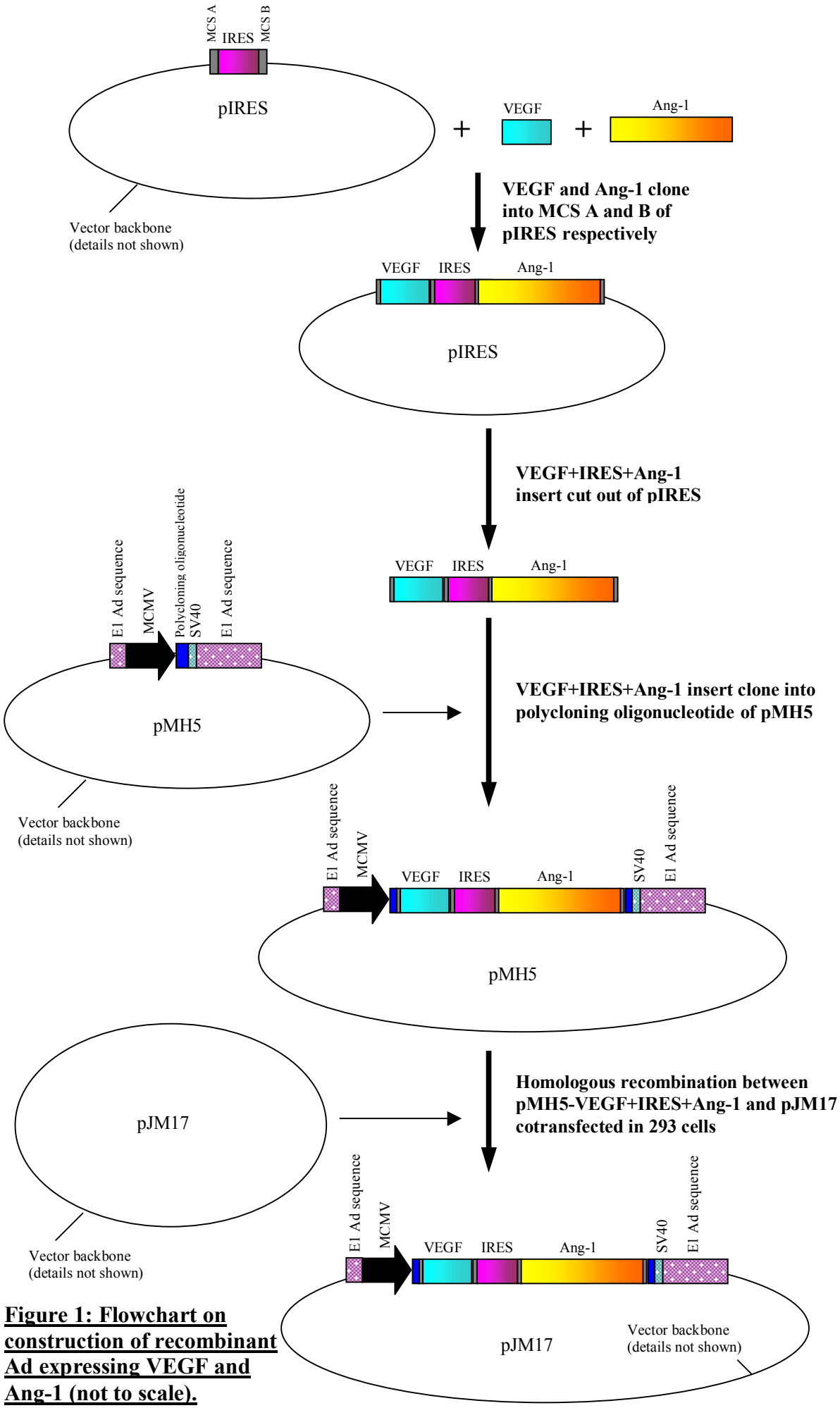

Figure 1: Flowchart on construction of recombinant Ad expressing VEGF and Ang-1 (not to scale).

1.5.3 Hypothesis

We hypothesized that the combo therapy approach based on skeletal myoblast transplantation together with angiogenic synergy between $VEGF_{165}$ and Ang-1 would provide beneficial effects to the ischemic limb in two ways. Whilst synergistic overexpression of $VEGF_{165}$ and Ang-1 genes would help to restore blood circulation in the ishemically damaged muscle fibers through neovascularizton in a localized and targeted fashion, concurrent neomyogeneis from the transplanted skeletal myoblast would give the additional therapeutic benefits to enhanced muscle function.

2 MATERIALS AND METHODS

All animals were subjected to humane treatment and all animal procedures were carried out in accordance with the Institutional Protocols and Guidelines approved by National University of Singapore and the Institutional Animal Care and Use Committee (IACUC) Singapore General Hospital. All biopsy and surgical procedures were carried out under anesthesia.

2.1 Skeletal myoblasts isolation and culture

For transplantation of autologous skeletal myoblasts transiently expressing $VEGF_{165}$ and Ang-1 genes to treat hind limb ischemia, skeletal myoblasts were obtained from the hind limbs of female adult New Zealand White rabbits (3 to 4.5 kg body weight).

2.1.1 Stimulation of skeletal muscle prior to biopsy

The site of skeletal muscle biopsy (femoral biceps muscle) was stimulated to induce migration of myoblasts to the site of injury/irritation by injecting 0.5 mL mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) at eight points, in and around the biopsy site.

2.1.2 Biopsy, myoblasts isolation and culture

Three days after stimulation of the biopsy site, the rabbit was prepared for removal of biopsy. The animal was anesthetized by intramuscular injection of ketamine (40 mg/kg body weight) and xylazine (8 mg/kg). For alleviation of pain, buprenorphine (0.03 mg/kg) and cephalexin antibiotics (15 mg/kg) were administered intramuscularly.

A skin incision was made on the hind limb, at the previously stimulated site of the femoral biceps muscle. Excisional biopsy of the first muscle layer of 1 to 3 g was removed and immediately immersed in a serum-free culture medium [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin] on ice. The incision was sutured and the animal was allowed to recover. The biopsy specimen was processed immediately under sterile tissue culture conditions for the isolation of myoblasts as reported by Qu et al. (2000) with slight modifications.

The muscle biopsy was sterilized in 70 % ethanol for 30 s and rinsed in serum-free culture medium. The muscle biopsy was minced into a coarse slurry followed by a series of enzymatic digestion using 0.2 % collagenase - type XI (C-9407; Sigma) for 90 min at 37 °C, 2.4 units/mL of dispase (GIBCO-BRL) for 45 min at 37 °C and finally 0.1 % trypsin-EDTA (Sigma) for 15 min at 37 °C. The muscle cell extract was pre-plated three times at 1 h time intervals to get rid of the debris and contaminating population of cells to increase the purity of myoblasts isolated from muscle biopsy. Briefly, the cells were resuspeneded in a patented Super-medium (Cell Transplants Singapore Pte. Ltd.) and plated onto three wells of a six-well tissue culture plate. The cells were allowed to settle and adhere to the base of the plate. Non-adherenet cells were transferred to the next

three wells of the plate after one hour of plating. The procedure was repeated three times before the plates were maintained in a humidified 5 % CO_2 atmosphere at 37 °C. Four days after seeding, the serum concentration of the culture medium (patented Super-medium) was increased by feeding the cells with Medium 199 supplemented with 20 % fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.1 mg/mL of gamma-irradiated L-glutamine. The cells were repeatedly passaged to prevent pre-mature differentiation and to achieve the required number for subsequent transplantation.

2.2 Desmin immunohistochemical staining

The purity of isolated myoblasts was evaluated by desmin immunohistochemical staining using the desmin immunohistology kit as per manufacturer's instructions (IMMH-5; Sigma). Brifely, skeletal myoblasts were first seeded onto polylysine coated sterilized glass slides and cultured to sub-confluence. They were air dried and fixed with acetone for 5 min at room temperature. The fixed cells were rehydrated twice in phosphate buffered saline (PBS) for 5 min each time. Endogenous peroxidase activity of the cells was quenched with 3 % hydrogen peroxide for 5 min at room temperature. The slides were washed in PBS for 5 min. Non-specific binding sites were blocked by incubating the slides with 1 % BSA for 15 min at 37 °C. Excess blocking solution was drained off and the cells were next incubated with biotinylated mouse monoclonal anti-desmin primary antibody for 2 h at 37 °C. After washing the slides twice with PBS for 5 min each, ExtrAvidin® - conjugated peroxidase was then added onto the slides and

incubated for 20 min at 37 °C. The slides were again washed twice with PBS for 5 min each and then dabbed dry. Finally, the peroxidase activity was revealed by applying AEC substrate reagent [a mixture of acetate buffer, 3-amino-9-ethylcarbazole (AEC) chromogen and 3 % hydrogen peroxide diluted in double distilled water] onto the slides. Adequate chromogen development was observed microscopically (Olympus-Japan) up to 20 min. When sufficient staining has been achieved, slides were rinsed in double distilled water and dabbed dry. Aqueous mounting media was applied onto the slides and then covered carefully with coverslip. The proportion of desmin positive myoblasts was then calculated from the ratio of cells with red stained cytoplasm and those unstained ones, obtained from counts of various microscopic fields.

2.3 Myoblasts labeling for cell fate monitoring

2.3.1 *nls*LacZ reporter gene labeling

Isolated skeletal myoblasts were transduced with *nls*LacZ reporter gene (LacZ reporter gene with nuclear localization signal) to allow monitoring of cell fate after transplantation.

2.3.1.1 Retroviral vector propagation and purification

Moloney Murine Leukemia Virus (MMLV) engineered to carry the bacterial *nls*LacZ gene, which encodes the bacterial enzyme β-galactosidase, was kindly gifted by A/P Reida Meshanwe El-Oakley. The virus was replication defective [by deletion of

sequences coding for essential proteins required for the packaging of the viral particles (Miller and Rosman, 1989; Vile et al., 1996)] and was packaged in FLY-A4 cells derived from human fibrosarcoma HT-1080 cells (American Type Culture Collection). FLY-A4 cells were cultured to confluence for 3 to 4 d in DMEM supplemented with 10 % FBS in a humidified, 5 % CO_2 atmosphere at 37 °C. The supernatant was then collected and filtered through a 0.2 μm filter.

2.3.1.2 nlsLacZ reporter gene retroviral transduction into myoblasts

*nls*LacZ reporter gene was retrovirally transduced into the skeletal myoblasts by incubating the cells with neat concentration of the filtered supernatant from FLY-A4 cells for 24 h in a humidified, 5 % CO_2 atmosphere at 37 °C. The transduction procedure was repeated twice consecutively for 2 d to achieve optimum transduction efficiency.

2.3.1.3 Histochemistry for β-galactosidase expression

To detect β-galactosidase expression, skeletal myoblasts transduced with *nls*LacZ were washed twice with PBS and fixed with 0.5 % glutareldehyde for 15 min at room temperature. After twice rinsing with PBS for 5 min each, the cells were incubated overnight at 37 °C with 40 mg/mL 5-bromo-4-chloro-3indoyl-β-D-galactopyranosid (X-gal; Bio-Rad), 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide and 2 mM magnesium chloride in PBS. At the end of the incubation period, the cells were rinsed in PBS and then observed under light microscope (Olympus-Japan) for bluish green stained nuclei.

2.3.2 BrdU labeling

Since *nls*LacZ retroviral transduction efficiency was less than 100 %, half of the isolated skeletal myoblasts were also double labeled with the thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) using the BrdU Labeling and Detection Kit II (Roche Molecular Biochemicals).

2.3.2.1 BrdU incorporation into myoblasts

BrdU was incorporated into skeletal myoblasts by incubating the cells with the BrdU labeling reagent (1:500 dilution with culture medium) for 48 h in a humidified, 5 % CO_2 atmosphere at 37 °C.

2.3.2.2 Immunohistochemical staining for BrdU

To ascertain for successful BrdU incorporation, skeletal myoblasts were grown on polylysine coated sterilized glass slides. The slides were air dried and fixed with absolute ethanol for 20 min at -20 °C. The cells were rehydrated twice in PBS for 5 min each. Following this, endogenous peroxidase activity was quenched from the tissues with methanolic hydrogen peroxide (1 part 3 % hydrogen peroxide to 4 parts of methanol) for 20 min at room temperature. The slides were washed twice in PBS for 5 min each and then dabbed dry. Non-specific binding was next blocked with Ultra-V blocking agent (LabVision) for 7 min at room temperature. Excess blocking solution was drained off and the cells were next incubated with mouse anti-BrdU primary antibody (1:100 dilution; Roche Molecular Biochemicals) for 30 min at 37 °C. At the end of the incubation period,

the slides were washed four times with PBS for 5 min each and then dabbed dry. Rabbit anti-mouse IgG-HRP conjugated secondary antibody (1:500 dilution; Chemicon) was then added onto the slides and incubated for 2 h at room temperature. The slides were again washed four times with PBS for 5 min each. Finally, the peroxidase activity was revealed by applying DAB (3,3'-diaminobenzidine) substrate reagent (LabVision) onto the slides. Adequate chromogen development was observed microscopically (Olympus-Japan) for 5 to 15 min. When sufficient staining has been achieved, slides were rinsed in double distilled water and dabbed dry. They were observed under light microscope for brown stained nuclei.

2.3.3 DAPI labeling

The other half of BrdU unlabeled cells were also double labeled with 4,6-diamidino-2-phenylindole (DAPI) to cover any unlabeled cells from the previous *nls*LacZ transduction procedures. This fluorescent dye binds to the double stranded DNA.

2.3.3.1 DAPI incorporation into skeletal myoblasts

The cells were trypsinized as per normal and the cell pellet was resuspended in 1 X DAPI [diluted from a 10 X stock solution (0.2 mg/mL DAPI; Sigma) with 0.9 % sodium chloride] for 40 min in a dark and humidified, 5 % CO_2 atmosphere at 37 °C.

Successful incorporation of DAPI into the cells was ascertained by observing the labeled cells under fluorescent microscope (Olympus-Japan) for blue fluorescent nuclei.

2.4 Adenoviral transduction of VEGF and Ang-1 genes into skeletal myoblasts

Isolated skeletal myoblasts were transduced with bicistronic Ad-vector carrying both $hVEGF_{165}$ and hAng-1 genes (Ad-Bicis), only $hVEGF_{165}$ gene (Ad-$VEGF_{165}$) or without angiogenic genes (Ad-Null). These viruses were kindly provided by Dr Ge Ruowen, Laboratory of Molecular Angiogenesis, National University of Singapore.

2.4.1 Adenoviral vector propagation and purification

Human adenovirus type-5 was used in this study. The virus was deficient for the early region 1 (E1) which was essential for its replication. As such, it was replication defective in all cells except in the E1-complementing 293 cells, a cell line derived from human embryonic kidney cells transformed by sheared Ad type 5 DNA.

Approximately three million 293 cells were seeded in a tissue culture treated flasks that have a growth surface area of 75 cm square (T-75) and this was allowed to grow for 4 d to reach confluence containing about 24 million cells. These cells were grown in DMEM supplemented with 10 % FBS and cultured in a humidified, 5 % CO_2 atmosphere at 37 °C.

At the end of the 4 d, Ad was propagated by infecting the confluent 293 cells. The thawed, cryopreserved virus was dispensed into the 293 monolayer that was covered with 20 mL of culture medium and this was then incubated for another 4 d in the humidified, 5 % CO_2 atmosphere at 37 °C till complete cytopathic effect was observed. Essentially, this is characterized by the change in the morphology of the cells from an attached, polygonal

appearance to a rounded, grape-like, detached one. The supernatant was then collected and filtered through a 0.2 μm filter. Ten milliliters of the filtered supernatant was mixed with 20 mL of culture medium and this was dispensed into a fresh T-75 that has already been seeded with 24 million 293 cells. Again, this was maintained for 4 d until complete cytopathic effect was observed. At the end of which the supernatant was collected and the above procedure of transferring the virus to a fresh 293 monolayer was repeated for subsequent maintenance of the Ad.

2.4.2 Adenoviral transduction of skeletal myoblasts

Primary myoblasts already expressing *nls*LacZ gene from a retroviral promoter and BrdU/DAPI labels, were transduced with either Ad-Bicis, Ad-VEGF or Ad-Null by incubating the cells with neat concentration of the filtered supernatant from 293 cells pre-infected with the respective Ad. This was maintained for 24 h in a humidified, 5 % CO_2 atmosphere at 37 °C. To achieve optimum transduction efficiency, this transduction procedure was repeated for a total of three times by replacing the used virus medium with fresh ones from the filtered supernatant of 293 cells (pre-infected with the respective Ad) and then maintaining it for another round of 24 h incubation.

2.4.3 Immunohistochemical staining for VEGF and Ang-1 expression

VEGF and Ang-1 genes overexpression from the transduced skeletal myoblasts was verified by either fluorescent or non-fluorescent immunostaining.

2.4.3.1 Double fluorescent immunostaining for VEGF and Ang-1 expression

In vitro Ang-1 and/or VEGF expression from Ad-Bicis and Ad-VEGF transduced myoblasts was detected with fluorescent immunostaining. Ad-Bicis or Ad-VEGF transduced skeletal myoblasts were grown on polylysine coated sterilized glass slides and cultured to subconfluence for about 2 to 5 d. They were then immunostained as described earlier in section 2.3.2.2 using rabbit anti-mouse Ang-1 (1:30 dilution; Chemicon) and/or mouse anti-human VEGF (1:50 dilution; PharMingen) primary antibodies. The reaction between the Ang-1 primary antibody and its antigen was visualized by using goat anti-rabbit IgG TRITC conjugated secondary antibody (1:100 dilution; Sigma) while those of VEGF was visualized by using goat anti-mouse IgG FITC conjugated secondary antibody (1:100 dilution; Sigma). The red (Ang-1) or green (VEGF) fluorescent signals emitted was then observed under a fluorescent microscope (Olympus-Japan). The cell nuclei were counterstained with mounting media containing DAPI (Vector shield).

The transduction efficiency of Ad-Bicis was obtained by calculating the proportion of VEGF positive myoblasts from the ratio of cells with green fluorescence to total number of cells, obtained from counts of 10 microscopic fields (Olympus-Japan). Similarly, the proportion of Ang-1 positive skeletal myoblasts was then calculated from the ratio of cells with red fluorescence to total number of cells, obtained from counts of various microscopic fields.

2.4.3.2 Non-fluorescent immunostaining for VEGF expression

In vitro VEGF expression from Ad-Bicis and Ad-VEGF transduced myoblasts was confirmed with non-fluorescent immunostaining. This was performed as described earlier in section 2.3.2.2 using mouse anti-human VEGF (1:50 dilution; PharMingen) primary antibody. The reaction between the VEGF primary antibody and its antigen was visualized by using biotinylated goat anti-polyvalent secondary antibody (LabVision) followed by streptavidin peroxidase (LabVision). The cells were sometimes counterstained with hematoxylin to visualize the nuclei.

The proportion of $VEGF_{165}$ positive myoblasts was then calculated from the ratio of cells with brown stained cytoplasm to total number of cells, obtained from counts of various microscopic fields.

2.4.4 Measurement of VEGF secretion

The pattern of transient VEGF gene expression was assessed by measuring the level of VEGF secreted into the culture medium using enzyme linked immunosorbent assay (ELISA). The culture medium containing the secreted VEGF protein, termed as 'conditioned medium', was collected every 48 h beginning from the last Ad transduction over a time period of 28 days. ELISA for $VEGF_{165}$ was performed using ChemiKine^{TM} Human VEGF Sandwich ELISA kit (Chemicon International, Inc.) as per supplier's instructions. Briefly, 100µl of each of the standards (5000 pg/mL, 1000 pg/mL, 200 pg/mL, 40 pg/mL and 0 pg/mL of VEGF standards) as well as the conditioned media obtained at different time points were dispensed into their respective wells pre-coated with mouse monoclonal antibodies generated against human $VEGF_{165}$. Each of the

conditioned media samples was pre-diluted with PBS prior to their addition into the wells. Twenty-five microliters of diluted biotinylated rabbit anti-human VEGF polyclonal antibody was then added into each well. The plate was sealed well to prevent evaporation and then incubated at room temperature for 3 h. Following this, each well was washed with 250 µL of wash buffer for a total of five times. Fifty microliters of diluted streptavidin-alkaline phosphatase was next dispensed into each well and incubated at room temperature for 45 min. The wells were again washed five times. Two hundred microlitres of colour reagent solution was dispensed into each well and the colour was allowed to develop at room temperature until the VEGF standard of concentration 5000 pg/mL reached an optical density (OD) reading of 1.6 to 1.8 OD units. The OD of each well was determined by using a microplate reader set to 490 nm wavelength with high shaking mode. Once this was achieved, 50 µL of stop solution was added to each well to stop the reaction. A final reading was then taken. The OD readings of the samples were compared to the log-log graph standard curve (Figure 2) generated from the VEGF standards to give the concentration of the VEGF protein detected in the samples.

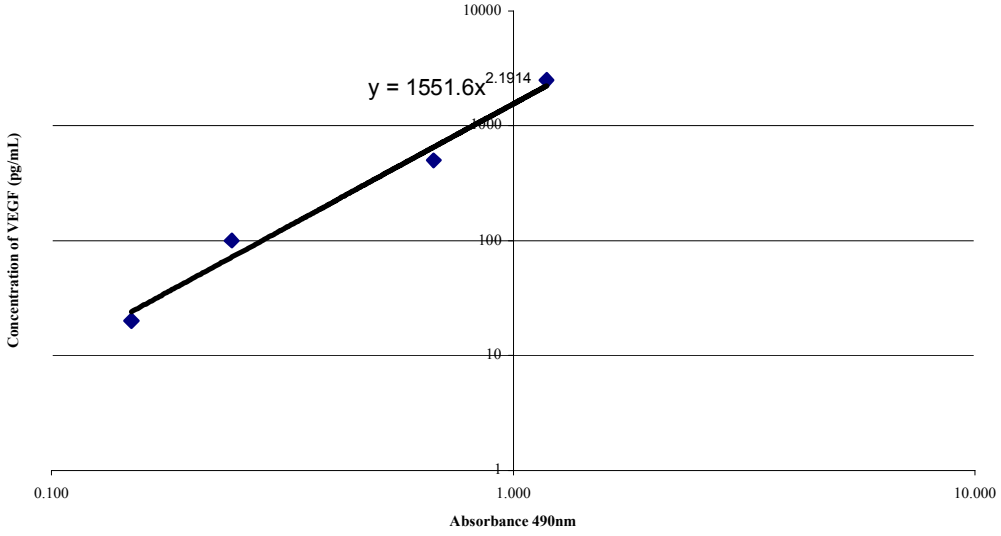

Figure 2. VEGF log-log standard curve

2.5 Hind limb ischemia model

2.5.1 Animal groupings

The animals were randomized into five groups to receive intramuscularly injected (i) DMEM group-1 (n=8), (ii) non-transduced myoblast transplanted group-2 (n=10), (iii) myoblasts transduced with Ad-Null group-3 (n=10), (iv) myoblasts transduced with Ad carrying $VEGF_{165}$ gene group-4 (n=10) and (v) myoblasts transduced with Ad carrying bicistronic VEGF and Ang-1 genes group-5 (n=10).

2.5.2 Development of animal model for hind limb ischemia

The animals were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and xylazine (8 mg/kg). Supplementary administration of the analgesic buprenorphine (0.03 mg/kg) and cephalexin antibiotics (15 mg/kg) were injected intramuscularly.

Femoral artery of the left hind limb was exposed and freed through a longitudinal incision from the inguinal ligament to a point just proximal to the patella. Multiple ligatures of the artery and all of its branches, beginning from the inguinal ligament right down to the point where it just bifurcates into the popliteal and saphenous arteries, were closed with Prolene suture 4-0 metric (Figure 3). Wounds were then closed.

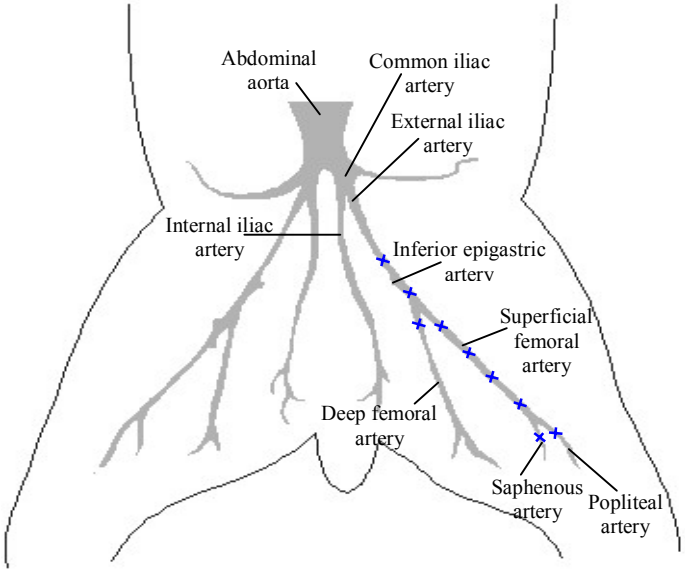

Figure 3. Rabbit hind limb ischemia model. Blue crosses show sites of ligations.

2.5.3 Confirmation on ischemic condition of animal model by angiography

As described earlier, the right femoral artery was then exposed and freed through a longitudinal skin incision. A 22 G intravenous catheter was inserted to infuse 2000 units of heparin followed by 10 mL of the iodinated contrast medium (Iomeprol, IOMERON® 350, Bracco). Serial filming of the left hind limb was done using Advantex angiography machine (General Electric, USA). Following this, the catheter was removed and the punctured artery was closed by ligation. Wounds were then closed.

2.6 Myoblast transplantation

In order to mimic the clinical setting more closely, inflammatory response after induction of hind limb ischemia was allowed to heal for approximately 10 d before skeletal myoblast transplantation, thereby simulating the treatment of acute hind limb ischemia.

A total of six to eight T-75 flasks confluent with skeletal myoblasts were trypsinized. They were labeled with *nls*LacZ and BrdU/DAPI and genetically modified with Ad-Null (group-3), Ad-VEGF (for group-4) or Ad-Bicis (for group-5) transduction. The old medium was first aspirated from the flask and the adhering cell monolayer was washed once with a sufficient volume of Hanks' Balanced Salt Solution. Trypsin/EDTA solution (1X) was then added to cover the adhering cell layer to allow for detachment of cells from the growth surface of the flask. The culture flask was checked for complete removal of the cells and cell suspension was centrifuged at 1500 rpm, 18 °C for 5 min in a refrigerated centrifuge. The supernatant was discarded while the cell pellet was washed twice with Hanks' Balanced Salt Solution before resuspending them in a total volume of 3 mL basal DMEM. The suspension was kept on ice for immediate transplantation.

The animals were anesthetized as earlier described and the tensor muscle of fascia lata, which was in close proximity with and along the projection of the femoral ligation site, was exposed through a longitudinal skin incision. Polypropylene sutures (Prolene 4-0 metric) were placed approximately 0.5 cm into the exposed muscle at four sites to permanently mark a rectangular site for injection. The cell suspension was then transplanted within this marked region through multiple injections of 100 μL volume each with the use of a 1-mL syringe and a 27-gauge needle. For each injection, the tip of the needle was inserted into the muscle at an angle of about 45° to its maximum allowable depth. The cell suspension was then slowly injected. For subsequent injections, the needle was withdrawn slowly and reinserted. For DMEM group, multiple injections of a total volume of 3 mL basal DMEM was injected instead of the cell suspension. After the final injection, the skin was sutured. This procedure of injecting myoblasts into an

exposed muscle gave much more reproducible results than did injecting the cells through the skin.

2.7 Post transplantation animal care

To avoid host immune rejection of the transplanted cells, animals in all the cell transplanted groups were immunosuppressed with 5mg/kg of cyclosporine on a daily basis 2 to 3 d before transplantation and thereafter two weeks after transplantation. The animals in all groups were also injected with the antibiotics cephalexin for up to 5 d after transplantation to counter any possible infection.

2.8 Angiography

Approximately eight weeks after transplantation, augmentation of collateral vessels in the ischemic limb was assessed by angiography.

The procedure followed that mentioned in section 2.5.3. Vascularization of the left thigh was quantified by direct counting of the number of arteries crossing 15 lines, drawn vertically across the midthigh [as reported by Pu et al. (1993) with slight modifications]. Vessel numbers from different animals within each group were summed up and averaged to obtain the angiographic score for that group. Collateral patterns were also observed.

2.9 Animal euthanasia

Animals were euthanized with an overdose injection of sodium pentobarbital through the marginal vein of the ear. Immediately after the sacrifice, the left hind limb was dissected and the region of transplantation, previously marked by the four sutures on the tensor muscle of fascia lata, was removed for histological studies.

2.10 Histological studies

Harvested tissues were dissected into small segments and each one was covered with O.C.T. compound before being snap frozen in partially thawed isopentane, previously cooled to –165 °C in liquid nitrogen. Multiple cryosections of 8 to 12 μm thickness were cut on a cryostat and placed on glass slides for histological studies.

2.10.1 Identification of transplanted skeletal myoblasts

Viability and identification of the transplanted donor skeletal myoblasts six to eight weeks after transplantation was established with X-gal staining for *nls*LacZ expression (12 μm thickness), BrdU staining (8 μm thickness) and DAPI fluorescence microscopic observation (12 μm thickness) on the muscle tissue sections. These were performed as mentioned in sections 2.3.1.3 (for *nls*LacZ), 2.3.2.2 (for BrdU) and 2.3.3.1 (for DAPI). For *nls*LacZ staining, sections were occasionally counterstained with hematoxylin to ascertain the position of nucleus in the tissue.

Whilst LacZ expression is well understood to be retained in cells even after multiple divisions for a long period of time (due to the gene being incorporated into the

host cell genome), BrdU and DAPI labels have been obseserved to be retained for up to six and eight weeks respectively. It must be noted, however, that DAPI fluorescence was emitted at a much weaker signal at eight weeks post-labeling compared to its earlier counterpart.

2.10.2 Capillary density and vessel maturation index quantification

To measure capillary density and vessel maturation index, the tissue sections (10 μm thickness) were stained for von Willebrand Factor VIII (vWF) and smooth muscle actin (SMA) to detect capillary endothelial cells and smooth muscle layer, respectively.

The tissue sections were immunostained as described earlier in section 2.4.3.1 using sheep anti-human vWF-FITC conjugated primary antibody (1:50 dilution; Acris) and mouse anti-α SMA primary antibody (1:100 dilution; Sigma). The reaction between the SMA primary antibody and its antigen was visualized by using rabbit anti-mouse-rhodamine conjugated secondary antibody (1:100 dilution; Chemicon) and observed under a fluorescent microscope (Olympus-Japan) for green (vWF) or red fluorescence (SMA).

For each tissue section, the number of capillaries was counted from four randomly selected microscopic fields under a 200x objective. A total of four different tissue sections from each animal, with two animals randomly selected per group, were used for measuring the final average capillary density. Vessel maturation index was calculated as the number of SMA-stained vessels over the total number of vessels within the field of view (obtained from the merged vWF and SMA stained vessel picture), counted under a 200x objective microscopic field.

2.10.3 Smooth muscle actin counterstaining on LacZ/DAPI positive sections

To observe for possible correlation between the transplanted region and any increase in blood vessel density, sections that were positive for extensive LacZ expression/DAPI fluorescence were also counterstained with SMA. For this, X-gal staining was first carried out as mentioned in section 2.3.1.3 or DAPI fluorescence was observed under fluorescent microscope, followed by SMA staining. This was performed as described earlier in section 2.3.2.2 using mouse anti-α SMA primary antibody (1:100 dilution; Sigma) primary antibody. The reaction between the α SMA primary antibody and its antigen was visualized using rabbit anti-mouse IgG-HRP conjugated secondary antibody (1:500 dilution; Chemicon) for non-fluorescent staining, or rabbit anti-mouse IgG-rhodamine conjugated secondary antibody (1:100 dilution; Chemicon) for fluorescent staining. For the latter, the slides were observed for red fluorescence under the microscope (Olympus-Japan).

2.10.4 T-lymphocytes immunohistochemical staining

Serial LacZ positive tissue sections were used for immunohistochemical staining to visualize T-lymphocyte infiltration at the site of the graft. Immunohistochemical staining was carried out using the protocol as detailed earlier in section 2.3.2.2 using mouse anti-rabbit T-lymphocyte-FITC conjugated primary antibody (1:50 dilution; Serotec). The reaction between the T-lymphocyte primary antibody and its antigen was visualized by using rabbit anti-mouse IgG-HRP conjugated secondary antibody (1:250 dilution; Chemicon) and revealed by DAB substrate. It was observed under a light microscope (Olympus-Japan) for brown stained cellular membrane.

2.10.5 Myosin heavy chain immunohistochemical staining

To observe for signs of possible neomyogenesis in the transplanted area, tissue sections that were positive for X-gal stain/DAPI fluorescence were counterstained for the skeletal muscle specific marker myosin heavy chain.

Immunohistochemical staining was carried out using the protocol as detailed earlier in section 2.3.2.2 using mouse anti-rabbit skeletal muscle myosin heavy chain primary antibody (1:100 dilution for non-fluorescent staining or 1:500 dilution for fluorescent staining; Sigma). The reaction between the myosin heavy chain primary antibody and its antigen was visualized by using rabbit anti-mouse IgG-HRP conjugated secondary antibody (1:500 dilution; Chemicon) and revealed by DAB substrate. For fluorescent staining, rabbit anti-mouse IgG-rhodamine conjugated secondary antibody (1:1000 dilution; Chemicon) was added and observed under a fluorescent microscope (Olympus-Japan) for red fluorescence.

2.10.6 VEGF immunohistochemical staining on LacZ positive sections

Tissue sections that were positive for X-gal stain were counterstained for VEGF to detect *in vivo* expression of this angiogenic growth factor. Staining procedure followed that as mentioned in section 2.4.3.2.

2.11 Statistical analysis

Using SPSS version 10.0 (SPSS Inc.), one-way ANOVA was used to determine if there exist any significant differences among the means of the different groups. Once this

was established, post-hoc pairwise multiple comparisons using the LSD (least significant difference) option were used to determine which are the means that differed. Normalcy of the data was tested using the Chi-square and one-sample Komolgorov-Smirnov tests.

3 RESULTS

3.1 Skeletal myoblasts isolation and culture

Besides skeletal myoblasts, the freshly isolated cells from rabbit hind limb skeletal muscle biopsy samples also contained a mixture of muscle-derived cells, including fibroblasts and adipocytes. Skeletal myoblasts were indicated by their typical cytoskeleton with larger spherical shape and having jagged edges (Figure 4, arrowheads). Blood cells and residual undigested muscle tissue were also present in culture. At day one, most of the cells were attached to the flask base, most of which acquired the spindle-shaped configuration typical of myoblasts while others retained their spherical shape with jagged edges. By day four, the cells started to form colonies. If left unpassaged, skeletal myoblasts multiplied until they reached confluence by day seven where they attained a particular wave-like alignment that radiates from a common origin. Thereafter, myogenic cells, which ceased dividing, commenced the *in vitro* fusion process when allowed to proliferate to over-confluence in low serum concentration, which resulted in formation of multinucleated tubes (Figure 4).

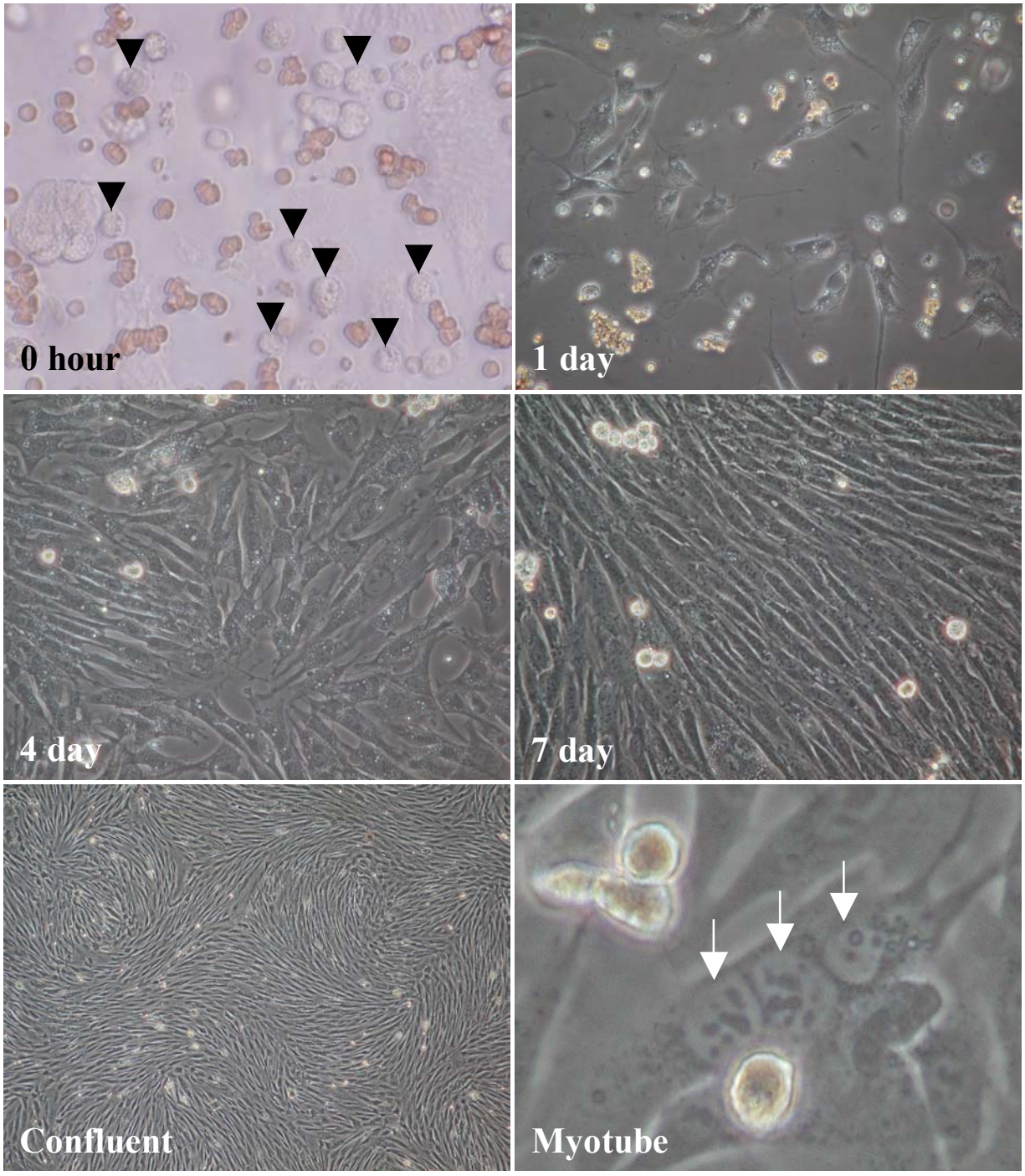

Figure 4. Phase contrast photomicrographs on morphology of rabbit primary myoblasts culture. Top and middle rows: Early culture of cells at 0h (400x magnification), 1d, 4d and 7d post-isolation (300x magnifications). Black arrowheads point to suspected myoblasts isolated. Bottom row: Late culture of cells at confluence phase (60x magnification) and an enlarged picture showing myotube formation, with white arrows indicating presence of multiple nuclei.

3.2 *In vitro* characterization of skeletal myoblasts

The population of muscle-derived cells after three times preplating and initial culture in the patented Supermedium displayed > 75-80 % positive desmin expressing cells (Figure 5). Primary skeletal myoblasts were transduced with the retroviral *nls*LacZ reporter gene and labeled with BrdU and DAPI to allow monitoring the fate of skeletal myoblasts after transplantation at various time points. *In vitro* retroviral transduction efficiency of skeletal myoblasts for *nls*LacZ reporter gene was more than 80 % as assessed by X-gal staining for β-galactosidase activity (Figure 6). BrdU and DAPI were also efficiently incorporated into the cells (Figure 6).

Skeletal myoblasts labeled with the three markers were also transduced with Ad-Bicis, only Ad-VEGF or Ad-Null vectors. Dual fluorescent immunostaining of skeletal myoblasts for $VEGF_{165}$ and Ang-1 after transduction with the Ad-Bicis revealed that more than 75-80 % of the cells simultaneously expressed both the growth factors (Figure 7). $VEGF_{165}$ expression was confirmed with non-fluorescent staining, revealed by DAB substrate (Figure 8). Cells transduced with the Ad-$VEGF_{165}$ also expressed the growth factor as revealed by fluorescent immunostaining for $VEGF_{165}$ (Figure 9). This was again confirmed with non-fluorescent immunostaining, revealed by DAB substrate (Figure 9).

The secretion level of $VEGF_{165}$ *in vitro* from the Ad-$VEGF_{165}$ transduced skeletal myoblasts was assessed by ELISA. $VEGF_{165}$ secreted into the culture medium was collected at a regular interval of 2 d, extending for a total period of 28 d of observation. The secretion level of $hVEGF_{165}$ from the Ad-Bicis transduced skeletal myoblasts peaked at 6 days post transduction (35.04±1.91 ng/mL) while that of Ad- $VEGF_{165}$ transduced skeletal myoblasts peaked at 4 days post transduction (19.52±4.12 ng/mL) (Figure 10).

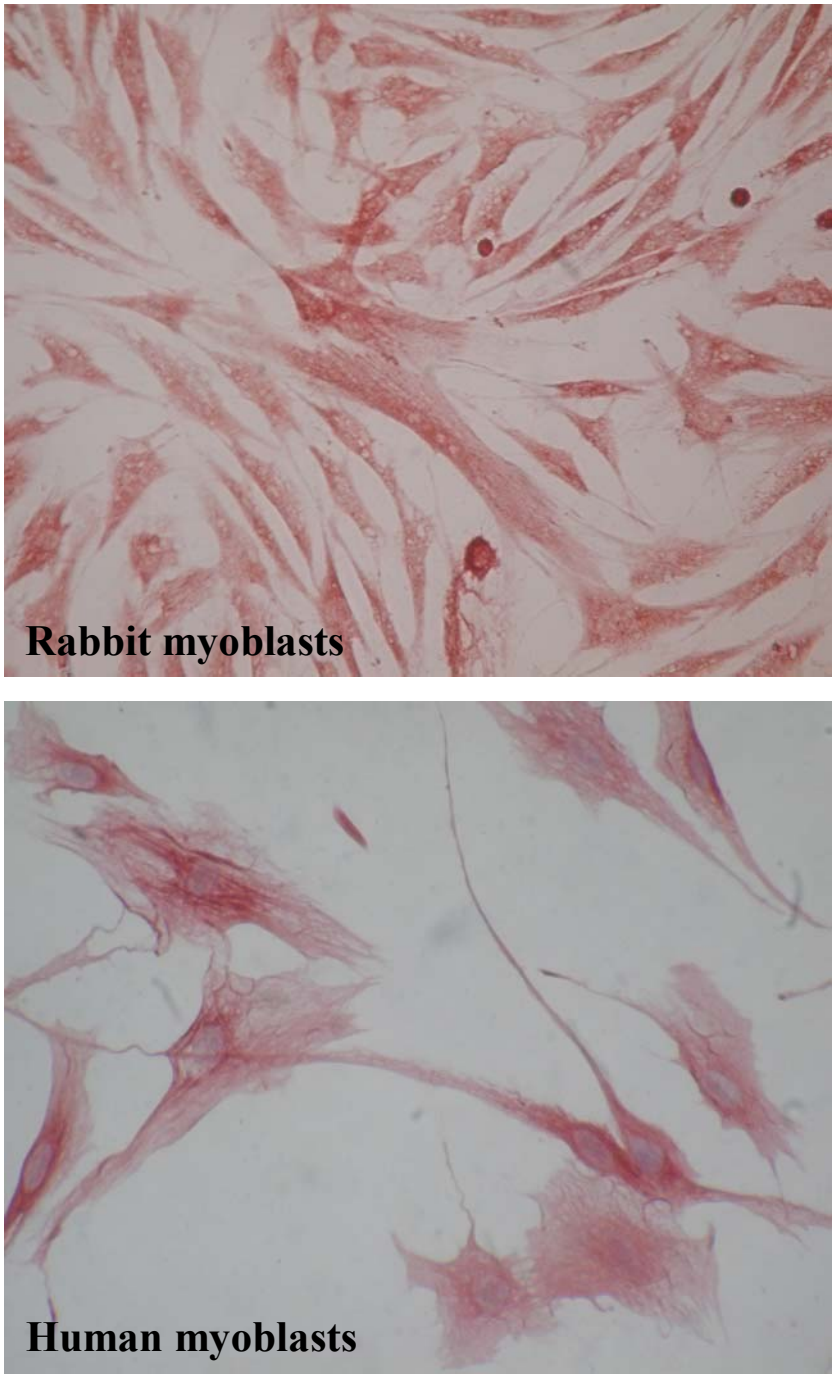

Figure 5. Desmin immunohistochemical staining to assess purity of myoblasts population isolated. Top: Rabbit primary myoblast population were > 75-80 % pure for desmin expression, revealed by AEC substrate (300x magnification). Bottom: Positive control using human myoblast cell line (Cell Transplants Singapore Pte. Ltd.), lightly counterstained with hematoxylin (400x magnification).

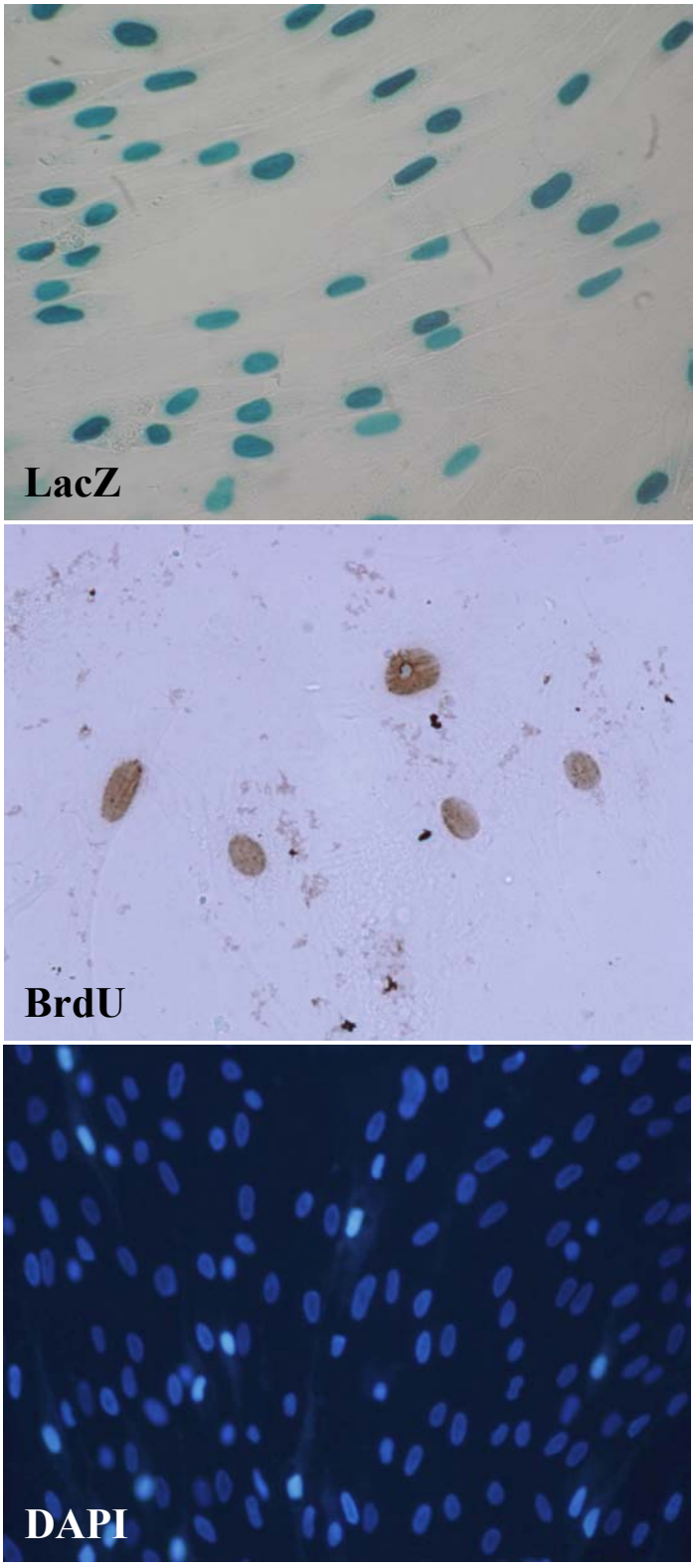

Figure 6. Nuclear labeling of rabbit myoblasts to allow monitoring of cell fate after transplantation. Top: *In vitro* retroviral transduction efficiency of myoblasts with *nls*LacZ reporter gene was about 80 %, as detected by X-gal staining (400x magnification). Middle: BrdU incorporated myoblasts detected by immunohistochemical staining (300x magnification). Bottom: DAPI labeled myoblasts observed under fluorescence (200x magnification).

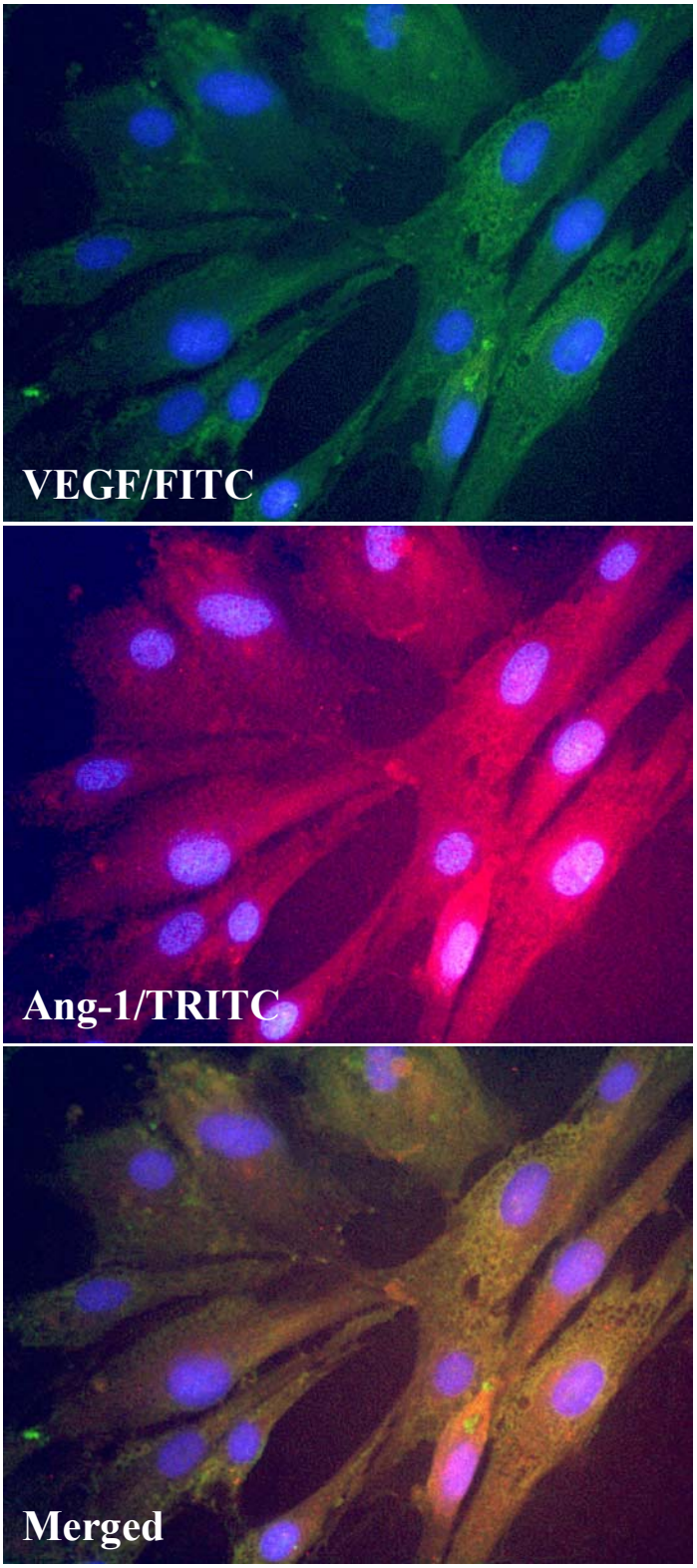

Figure 7. Dual immunofluorescent staining of Ad-Bicis transduced myoblasts to detect $VEGF_{165}$ and Ang-1 expression. More than 75-80 % of the cells simultaneously expressed both growth factors**.** Top: FITC fluorescence of $VEGF_{165}$ expressing myoblasts overlayed with DAPI fluorescence. Middle: Corresponding TRITC fluorescence of Ang-1 overlayed with DAPI fluorescence. Bottom: Merged fluorescence of $VEGF_{165}$ Ang-1 and DAPI. (300x magnifications)

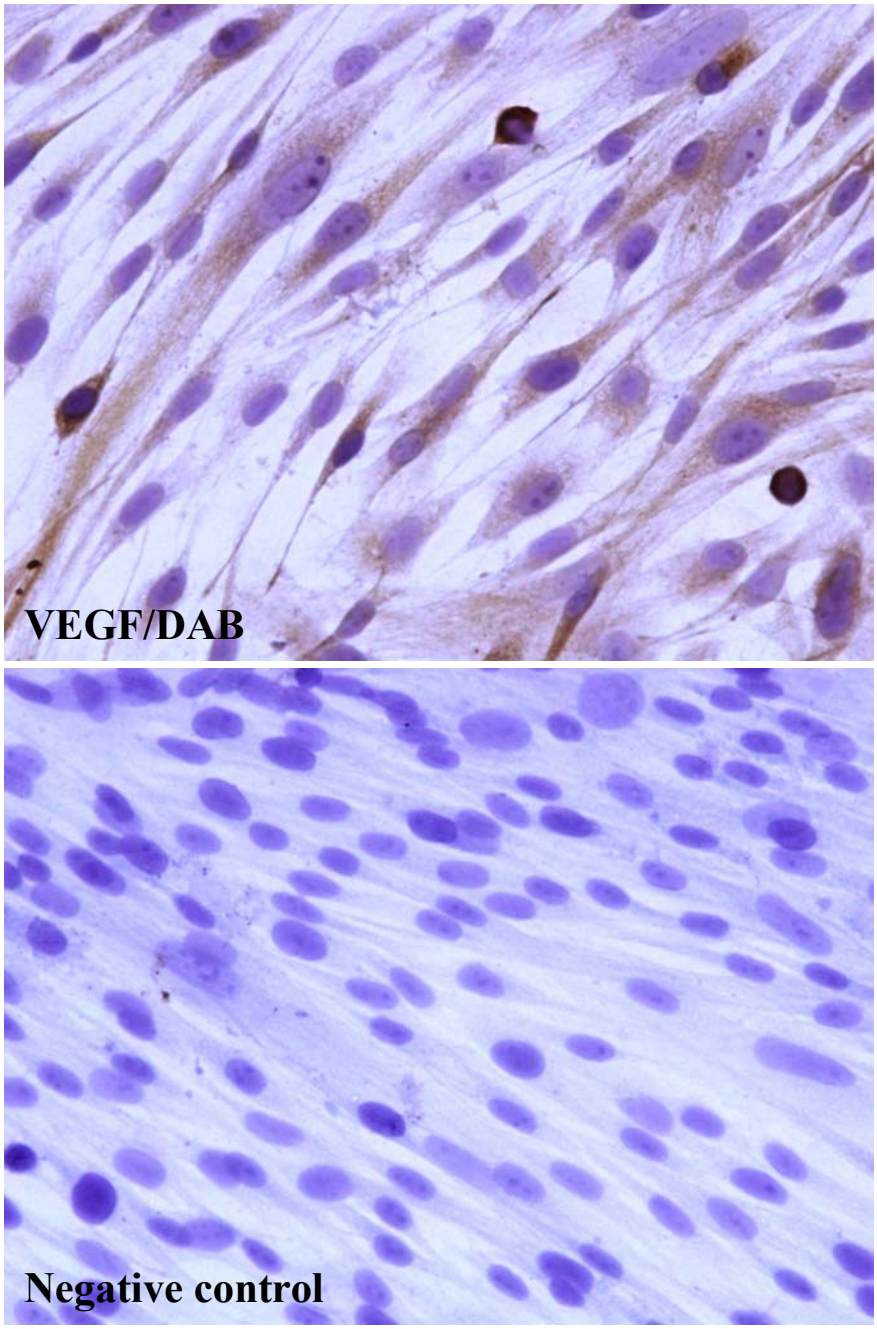

Figure 8. $VEGF_{165}$ immunohistochemical staining of Ad-Bicis transduced myoblasts. Top: $VEGF_{165}$ expression from Ad-Bicis transduced myoblasts was confirmed with non-fluorescent staining, revealed by DAB substrate, and hematoxylin counterstaining. Bottom: Negative control with primary antibody omitted, and counterstained with hematoxylin. (300x magnifications)

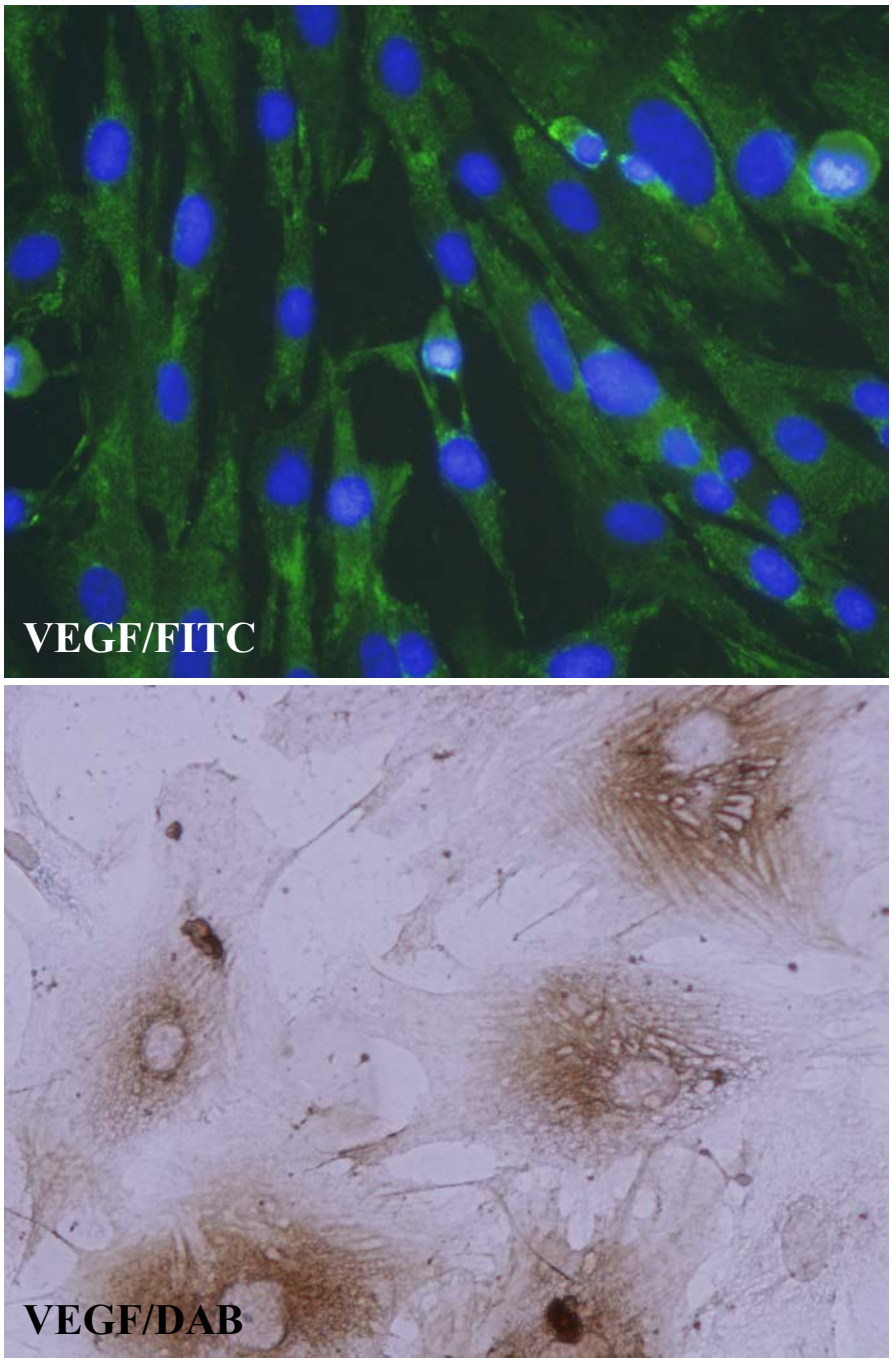

Figure 9. $VEGF_{165}$ immunohistochemical staining of Ad-VEGF transduced myoblasts. Top: FITC fluorescence of $VEGF_{165}$ expressing myoblasts overlayed with DAPI fluorescence. Bottom: $VEGF_{165}$ expression confirmed with non-fluorescent staining, revealed by DAB substrate. (300x magnifications)

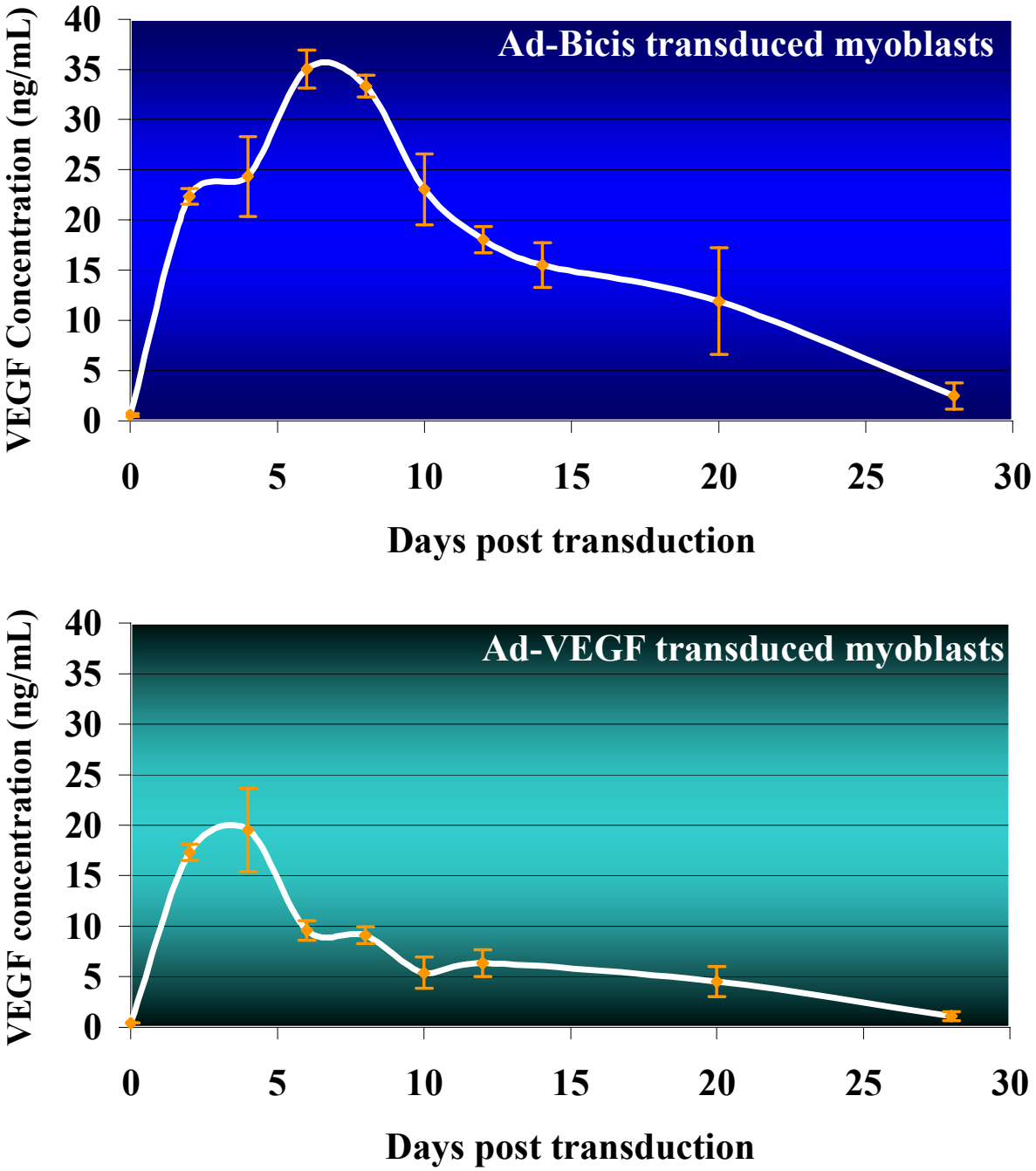

Figure 10. Measurement of $VEGF_{165}$ secretion from Ad transduced myoblasts by ELISA. Top: Level of $VEGF_{165}$ secreted from Ad-Bicis transduced myoblasts peaked at 6 days post transduction (35.04±1.91 ng/mL). Bottom: Level of $VEGF_{165}$ secreted from Ad-VEGF transduced myoblasts peaked at 4 days post transduction (19.52±4.12 ng/mL).

3.3 Fate of transplanted skeletal myoblasts

Following transplantation into rabbit ischemic skeletal muscles, extensive survival of skeletal myoblasts was observed at the site of the graft (Figure 11-14). A proportion of *nls*LacZ positive donor skeletal myoblasts successfully integrated into mature or regenerating host muscle fibers to form heterokaryons. The donor skeletal myoblast nuclei continued to express *nls*LacZ reporter gene as observed by histochemical staining for β-galactosidase activity. Fusion to form mature host fibers was evident from the strictly peripherally located green donor nuclei that were interspersed and aligned with the host purple nuclei as visualized by counterstaining with hematoxylin. This was further confirmed by immunostaining for BrdU and observing for DAPI fluorescence (Figure 11). On the other hand, fusion to regenerating host muscle fibers was indicated by presence of some centrally located *nls*LacZ donor nuclei (Figure 12). The other fates of the transplanted myoblasts include fusion to other implanted myoblasts (Figure 13), remained quiescent as mononucleated cells (Figure 14) or death. One possible cause of death of these transplanted myoblasts is host immune rejection as revealed by presence of infiltrating T-lymphocytes in ischemic sites of cell grafting (Figure 15).

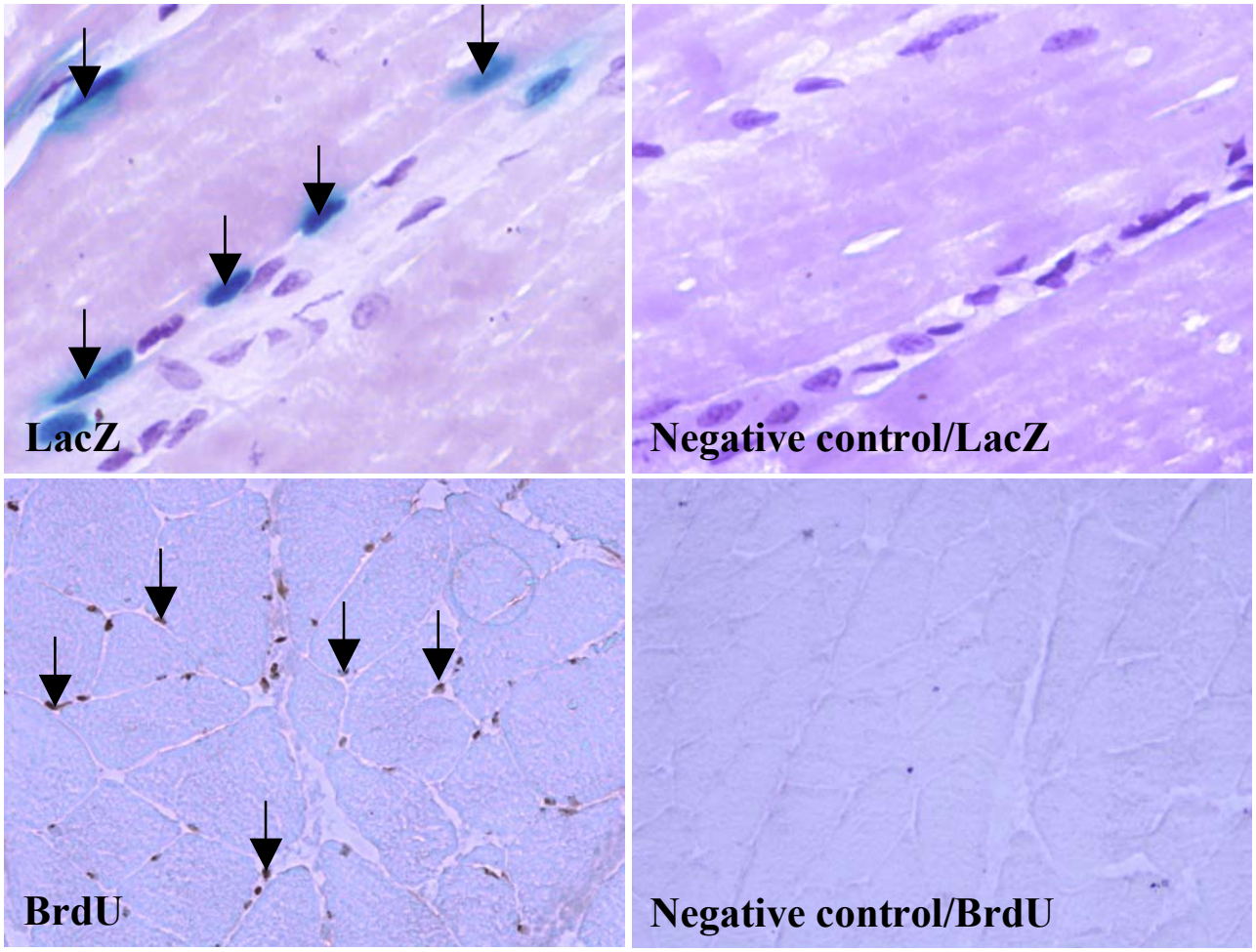

Figure 11. Fate of transplanted skeletal myoblasts - fusion to mature host fibers. Top row: *nls*LacZ expressing donor myoblasts (green nuclei) in the host tissue of group-3 at 6 w after transplantation as revealed by X-gal staining, and counterstained with hematoxylin to visualise host nuclei (purple). Tissue section from DMEM injected group at 8 w post-treatment was used as negative control, also counterstained with hematoxylin. (600x magnifications) Middle row: Immunohistochemical staining on tissue section from group-4 at 6 w after transplantation for BrdU positive donor myoblasts, revealed by DAB substrate (300x magnification). Negative control with primary antibody omitted (150x magnification). Arrows indicate representative transplanted cells. Continued in following page.

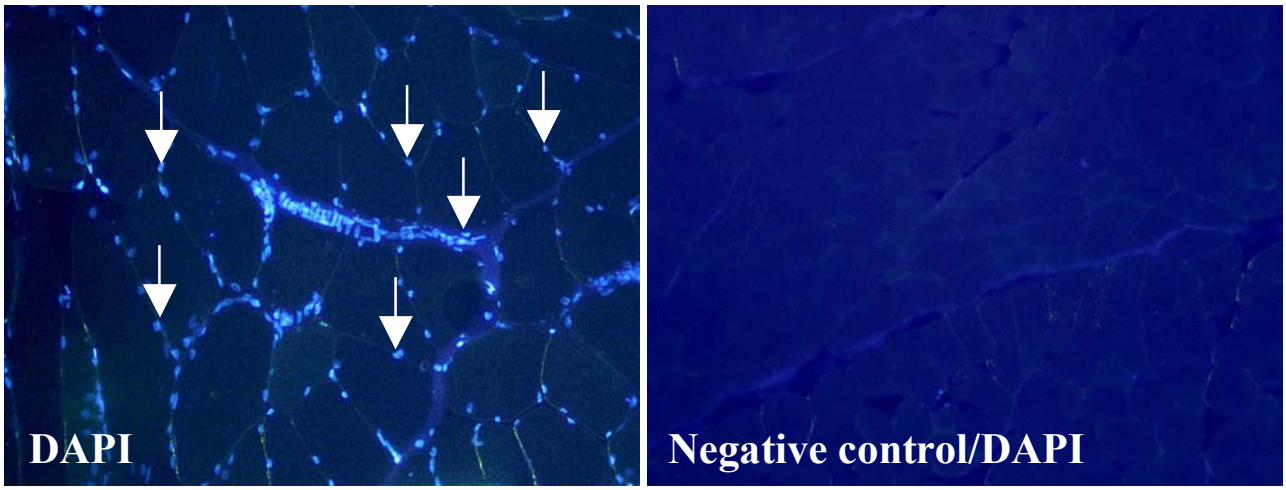

Figure 11 continued. Fate of transplanted skeletal myoblasts – more evidence of fusion to mature host fibers. DAPI labeled donor myoblasts observed under fluorescence microscopy, in group-5 at 8 w after transplantation confirmed fusion to host fibers (150x magnification). Absence of DAPI fluorescence in tissue section from DMEM injected group at 8 w post-treatment used as negative control (100x magnification). Arrows indicate representative transplanted cells.

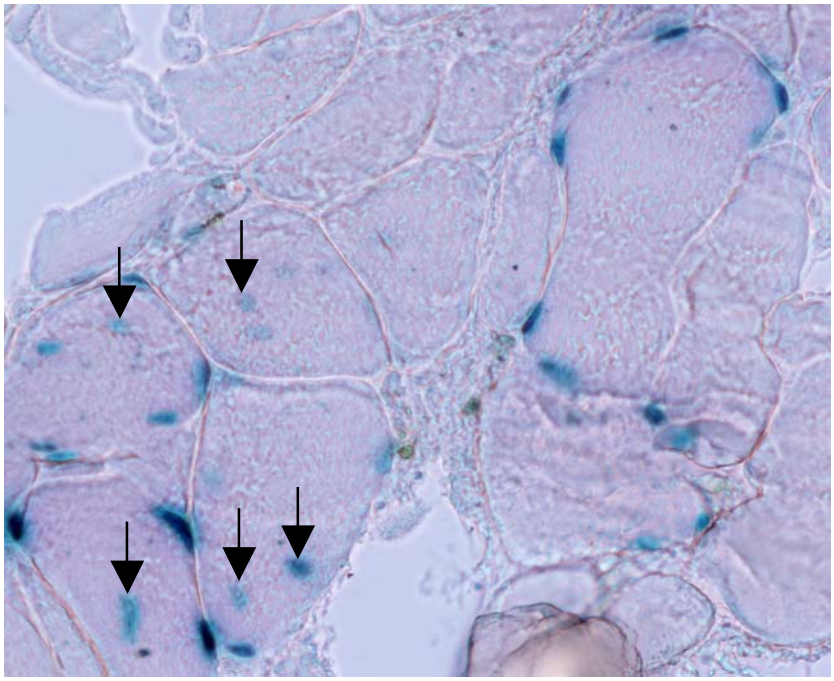

Figure 12. Fate of transplanted skeletal myoblasts – fusion to regenerating host fibers. LacZ expressing donor myoblasts (green nuclei) at site of grafting in group-2 at 6 w after transplantation as revealed by X-gal staining (200x magnification). A proportion of these green donor nuclei were centrally located in the muscle fiber, suggesting their fusion to regenerating fibers. Arrows indicate representative centrally located donor nuclei.

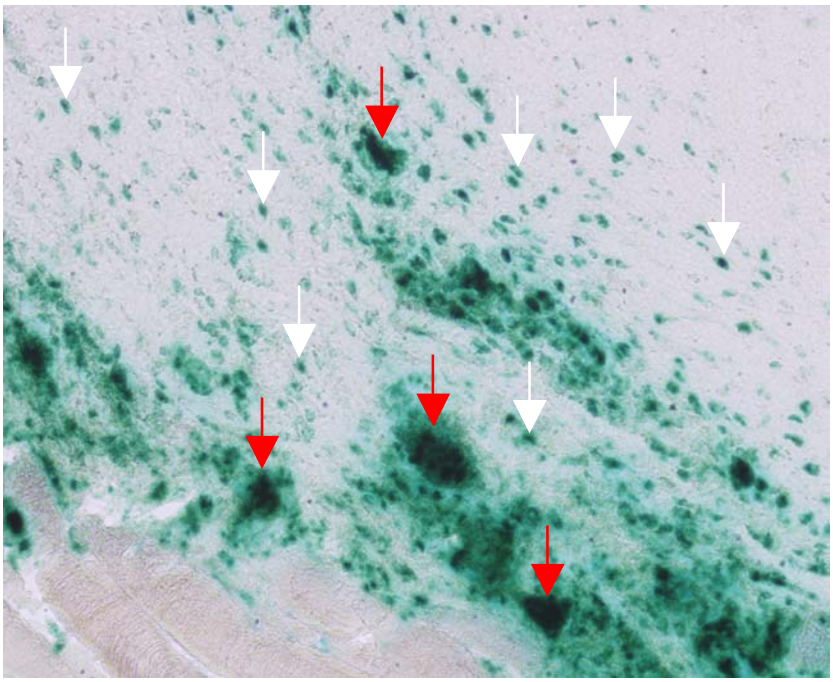

Figure 13. Fate of transplanted skeletal myoblasts – fusion to other implanted myoblasts. LacZ expressing donor myoblasts (green nuclei) in an ischemic region of the limb in group-4 animal, as revealed by X-gal staining at 6 w after transplantation (100x magnification). The mass of β-galactosidase expression at certain sites (indicated by red arrows) possibly involved fusion amongst implanted myoblasts. White arrows point to other implanted myoblasts that have remained quiescent as mononucleated cells.

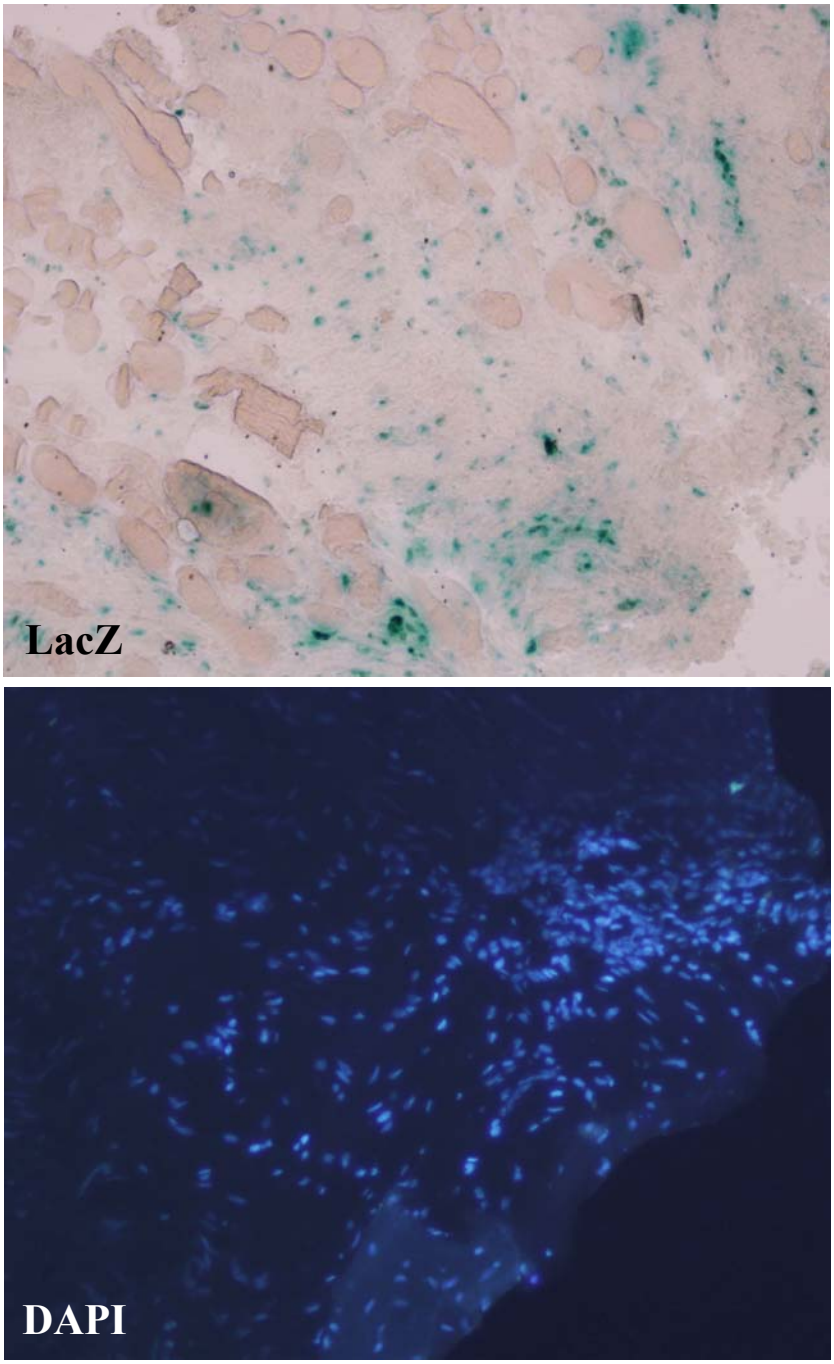

Figure 14. Fate of transplanted skeletal myoblasts – remained quiescent as mononucleated cells. Top: LacZ expressing donor myoblasts (green nuclei) remained quiescent as mononucleated cells in-between degenerating muscle fibers of an ischemic area of a group-2 animal, as revealed by X-gal staining at 6 w after transplantation (100x magnification). Bottom: Confirmation by presence of haphazardly arranged DAPI labeled cells in another ischemic region of a group-4 animal at 6 w after transplantation (150x magnification).

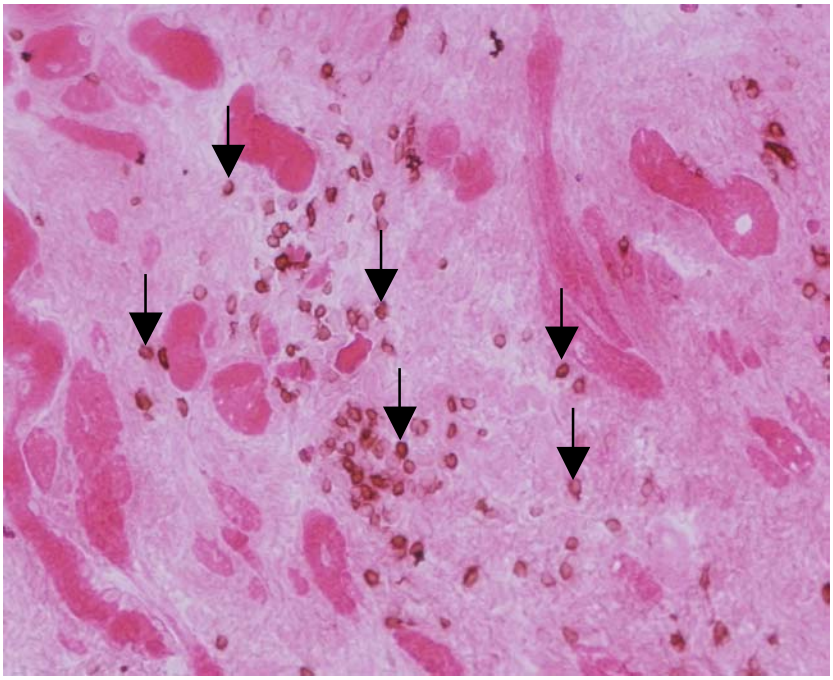

Figure 15. T-lymphocyte immunohistochemical staining to detect immune rejection of transplanted skeletal myoblasts. Site of cell grafting (not shown) at an ischemic region of a group-4 animal at 6 w after transplantation revealed presence of infiltrating T-lymphocytes, suggesting death of transplanted myoblasts by host immune rejection. Section was counterstained with eosin to visualize muscle fiber architecture (200x magnification). Arrows indicate representative T-lymphocytes revealed with DAB substrate.

3.4 Animal model preparation and angiographic assessment

Partial ischemia was induced in left hind limb of the rabbits by surgical ligation of the external iliac artery and superficial femoral artery, leaving the internal iliac artery intact. Eight-point permanent ligation of the external iliac and femoral arteries was carried out to achieve complete occlusion of the blood flow to produce sufficient ischemia (Figure 16). In addition, contrary to most previously documented studies that excised a substantial part of the femoral artery, we modified the procedure of animal model development by occluding a short sequence of this vessel that was sufficiently long to avoid simple 'jumping' of collaterals across the ligature in order to make it clinically more relevant and with similarity to the natural obliterative disease process. Similar to studies that developed animal models of hind limb ischemia by excision of the femoral artery, our animal model demonstrated complete occlusion of the ligated blood vessel and its tributaries, as revealed by angiographic assessment at 10 d post ligation. Several radiographically visible corkscrew collaterals arising from the internal iliac artery were also observed in the limb at this time point (Figure 16A-E), and this appears to be more than in the nonoccluded hind limbs (Figure 16). Despite such collateral growth in the thigh, perfusion in the lower leg does not reach maximal normal values (Schaper et al., 1976), thus leaving substantial room for therapy. We also witnessed occasional gangrene and gross impairment of function (shown most obviously by limping and the appearance of skin necrosis, and necrotic black toes) within a short period after the ligation procedure, thus confirming the success of the model development.

At 6 w after treatment, significantly increased angiographic collateral blood vessels count was observed in group-5 (25.14±5.14) as compared to group-2 (4.67±3.49; P=.024)

or DMEM-injected group-1 (3.18±7.76; *P*=.022) (Figure 17F-K). These collateral blood vessels represented a network of newly formed arteries as they were not detected before treatment. They were distributed in a broad area of the ischemic muscles sprouting from the internal iliac artery towards the distal medial thigh region. However, angiographic score from the Ad-VEGF myoblast transplantation in group-4 (13.62±4.52; *P*=.181) or Ad-Null myoblast taransplantation in group-3 (6.09±0.09; *P*=.057) were not significantly different from the scores of group-5, though group-5 still sustained the highest value amongst all groups (Figure 17K). In addition, several newly formed collateral vessels were also detectable in the DMEM injected control group-1 (Figure 17J). This is similar to a previous report by Cao et al. (2003) who stated that such a compensatory effect of ischemia-induced collateral growth was normally observed in models 6 to 8 w after ligation.

There were no animal deaths related to the skeletal myoblast transplantation procedure. On the whole, all animals completed the entire study duration successfully, albeit occasional problem of infection which arose due to re-opening of the ligation wounds.

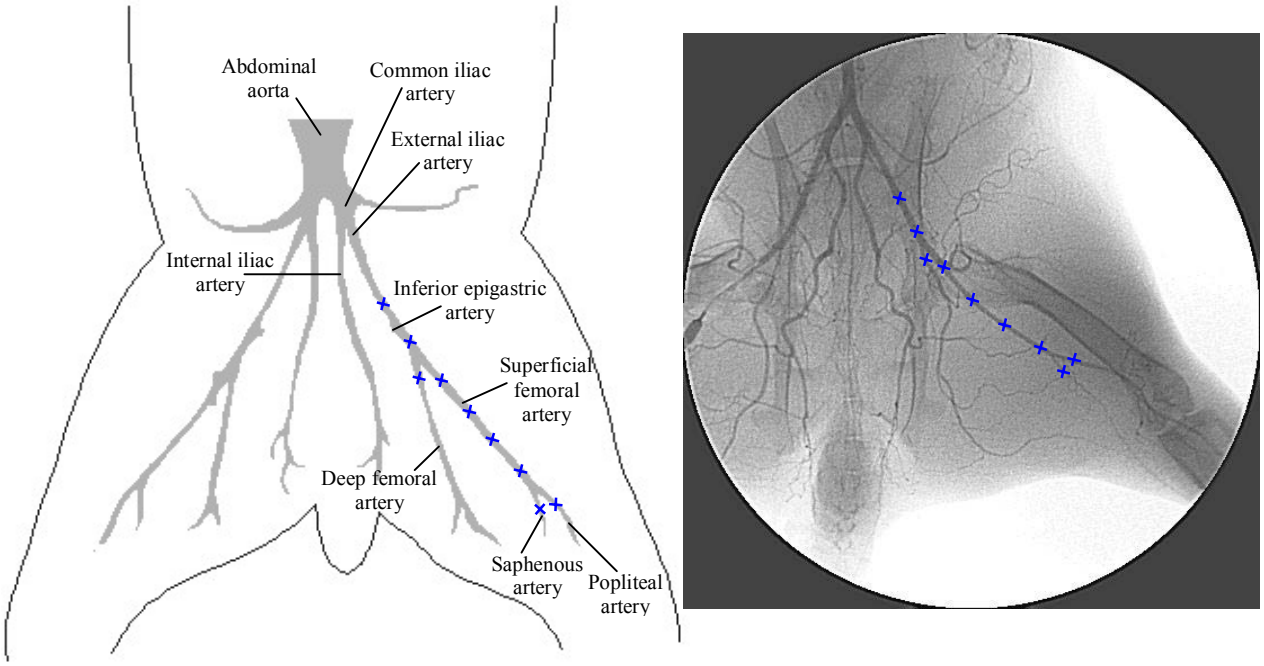

Figure 16. Creating rabbit model of left hind limb ischemia. Left: Schematic diagram showing sites of multiple ligations (blue crosses) of left femoral artery, beginning from inguinal ligament right down to where it bifurcates into the popliteal and saphenous arteries. Right: Angiogram on normal rabbit hind limb before ligation. Again, blue crosses show sites to be ligated.

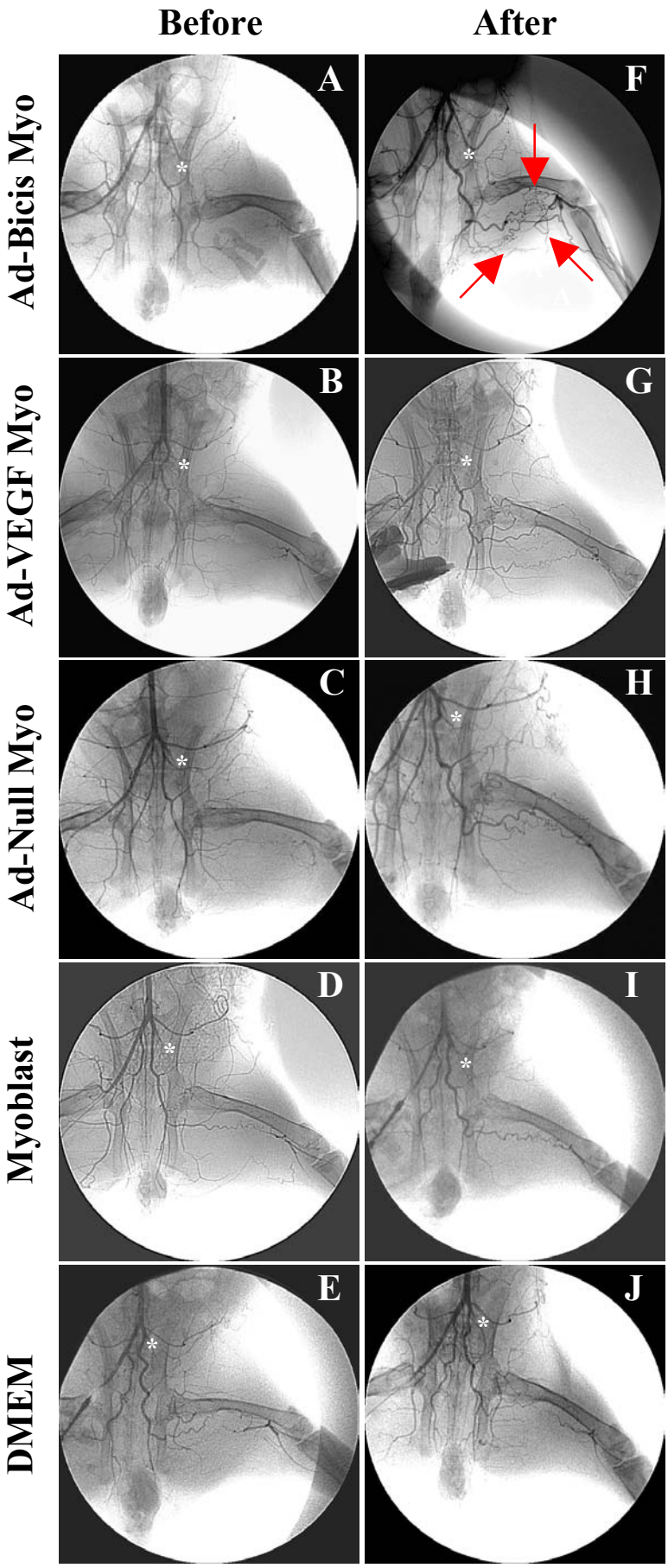

Figure 17A-J. Angiographic assessment to detect collateral formation.
Left column (A-E): Baseline angiograms at 10 days post-ligation and before treatment.
Right column (F-J): Angiograms of the same corresponding rabbits 6 w after intramuscular injection of either Ad-Bicis transduced myoblasts, Ad-VEGF transduced myoblasts, Ad-Null transduced myoblasts, non-transduced myoblasts or only DMEM.
White asterisks mark ligation sites. Red arrows point to region of increased collaterals.

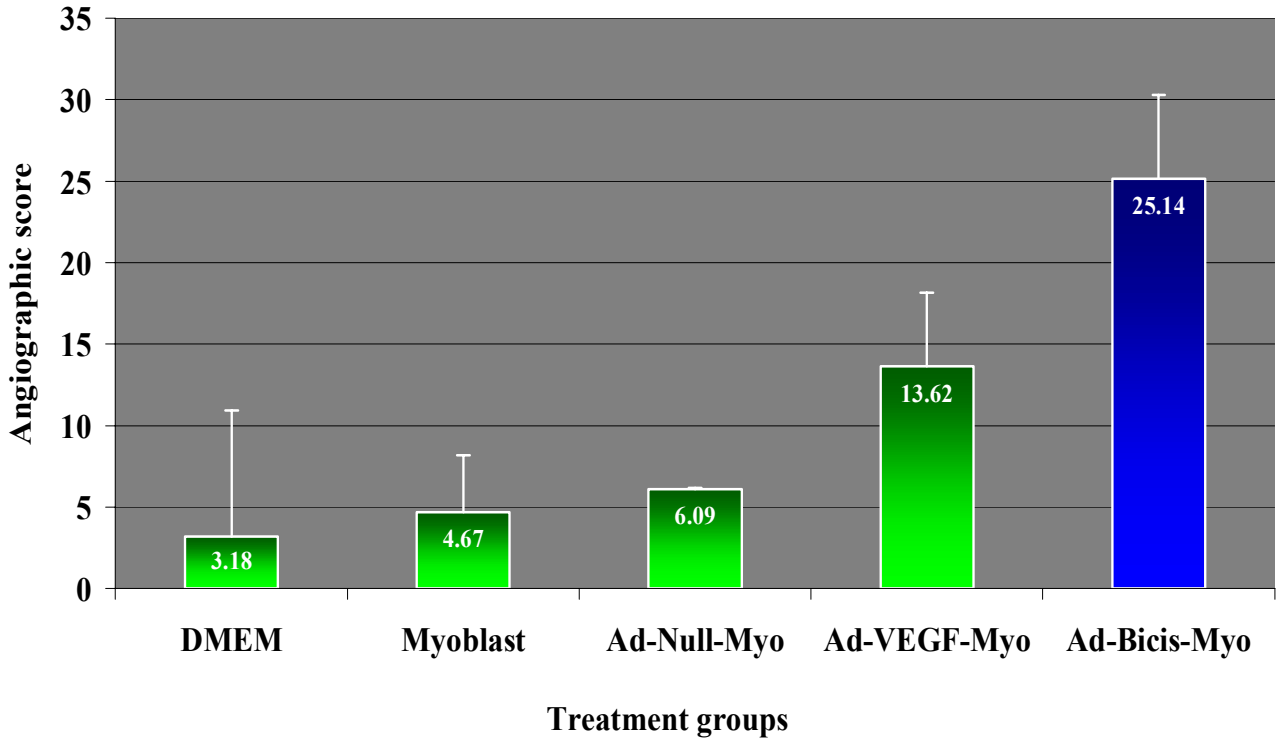

Figure 17K. Angiographic score from quantification of collaterals in different treatment groups. Angiographic blood vessel count revealed enhanced neovascularization in Ad-Bicis transduced myoblasts treated group. *P*=.022 vs DMEM; *P*=.024 vs Myoblast; *P*=.057 vs Ad-Null Myo; *P*=.181 vs Ad-VEGF Myo.

*DMEM group-1 (n=4), Myoblast group-2 (n=4), Ad-Null Myo group-3 (n=2), Ad-VEGF Myo group-4 (n=4), Ad-Bicis Myo group-5 (n=7)

3.5 Blood vessel density assessment

As the angiographic analysis detects only relatively larger size collateral vessels and not the finer capillary network, a double immunolocalization using fluorescent vWF and SMA antibodies was performed. It revealed much superior capillary density [number of vWF-stained capillaries/high power microscopic field (200x)] in group-5 (19.04±1.59) as compared to group-4 (15.31±1.55; *P*=.009), group-3 (6.53±0.97; *P*=.000), group-2 (5.69±0.51; *P*=.000) and group-1 (3.03±0.20; *P*=.000) (Figure 18A-P).

A large proportion of blood vessels in group-5 possessed a thick smooth muscle cell coating as revealed by the merged images of vWF and SMA, thus suggesting the maturation of these newly formed vessels (Figure 18E, J, O). This was further confirmed using non-fluorescent DAB chromogenic immunohistochemical staining for SMA (Figure 19A-B). It revealed a more detailed observation of numerous fine, small vessels surrounding the periphery of muscle fibers, albeit at a weaker stain. However, such high density of mature vessels were also observed in the other treatment groups as calculated by their similar maturation index [number of SMA-stained vessels / total number of vessels obtained from the merged vWF and SMA images, counted under a high power microscopic field (200x)] (Figure 18Q). We did not observe formation of hemangiomas in any of the treatment groups. In addition, DAPI or LacZ expressing donor skeletal myoblasts and SMA positive vessels showed a proportional degree of expression, be it in ischemic (Figure 20-21) or viable muscle area (Figure 22). At the site of extensive DAPI/Lac-Z expression, a corresponding increase in SMA positive vessels was also observed, whilst at a contralateral region where minimal DAPI/LacZ expression was detected, a corresponding low number of SMA positive vessels were found.

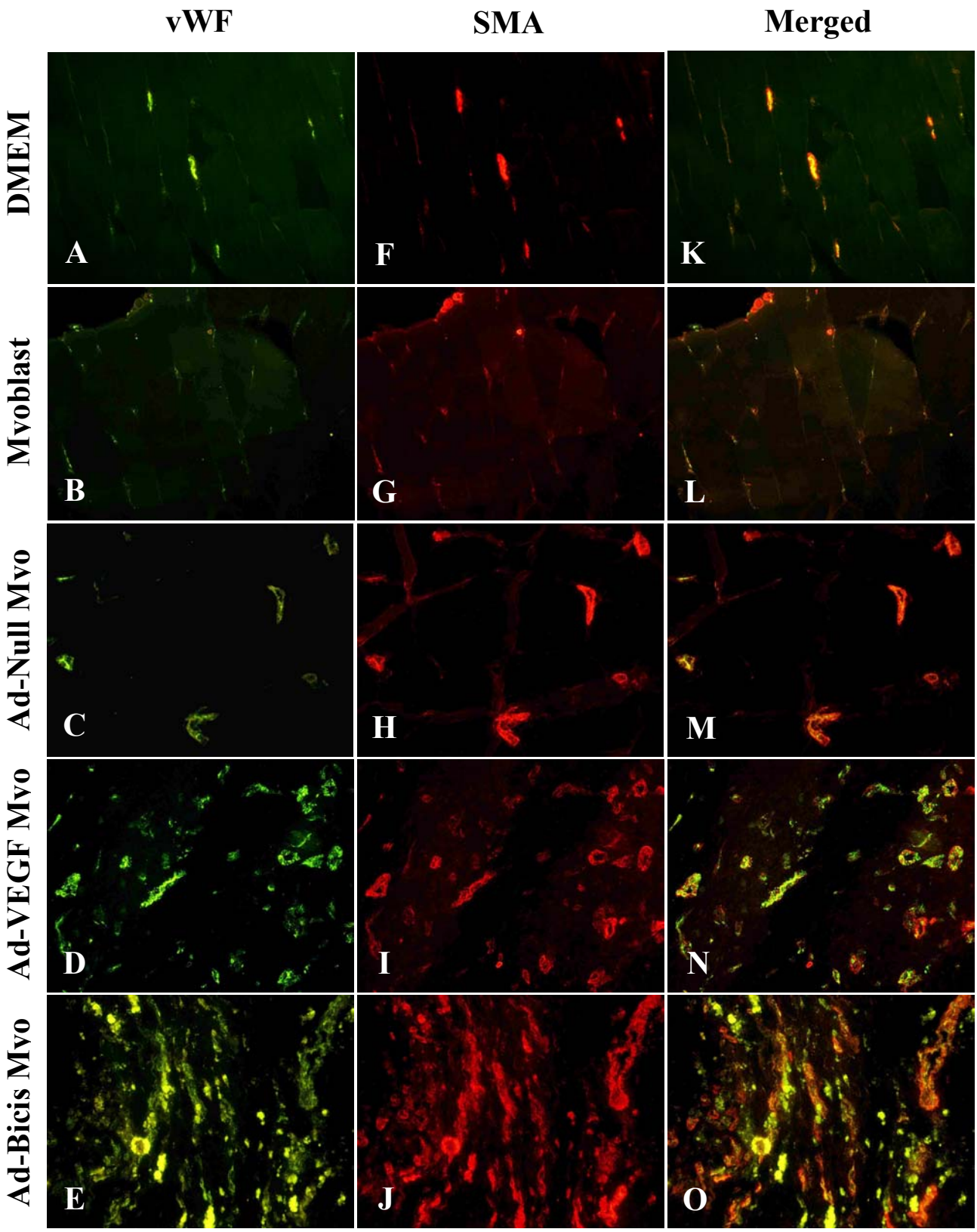

Figure 18A-O. Double immunolocalization using fluorescent antibodies against vWF and SMA to detect blood vessel density. Left column (A-E): FITC fluorescence of vWF expression from ischemic limb tissue of DMEM, myoblasts, Ad-Null transduced myoblasts, Ad-VEGF transduced myoblasts and Ad-Bicis transduced myoblasts treated groups. Middle column (F-J): Corresponding TRITC fluorescence of SMA expression from the respective treatment groups. Right column (K-O): FITC and TRITC overlayed fluorescence of vWF and SMA expression respectively. (200x magnifications)

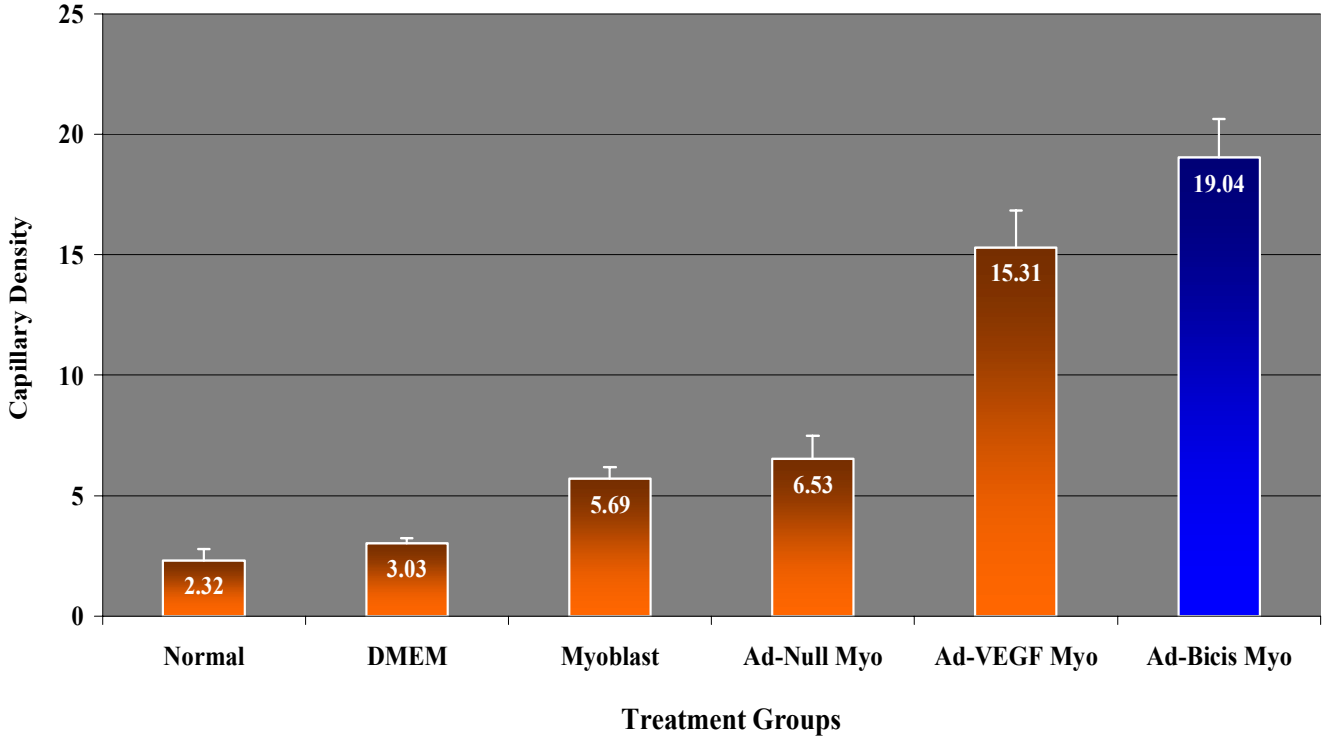

Figure 18P. Capillary density quantitated from vWF positive vessels in different treatment groups. Capillary density was significantly highest in the Ad-Bicis Myo group (P<0.001 vs normal, DMEM, Ad-Null Myo and Myoblast groups. P=.009 vs Ad-VEGF Myo group).

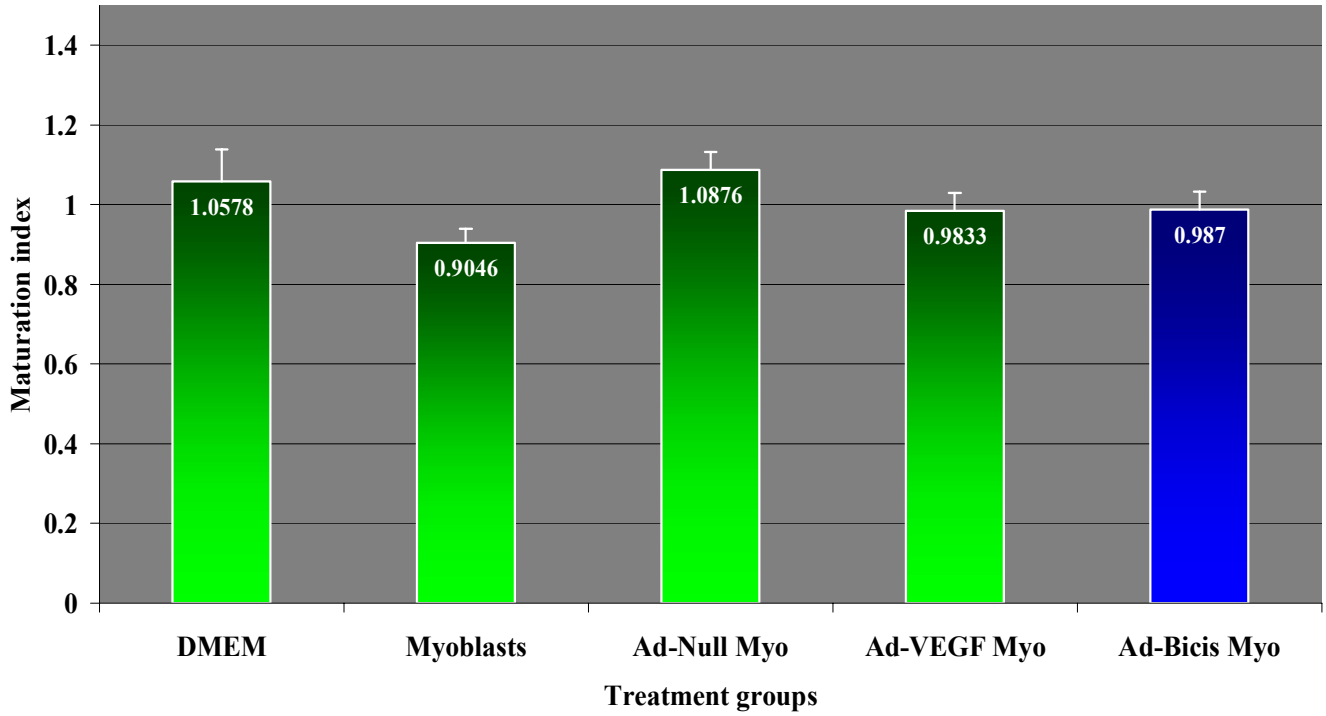

Figure 18Q. Maturation index of blood vessels formed in different treatment groups. Majority of the blood vessels in all groups appear mature, with no significant difference in maturation indices found across all groups (P>.05).

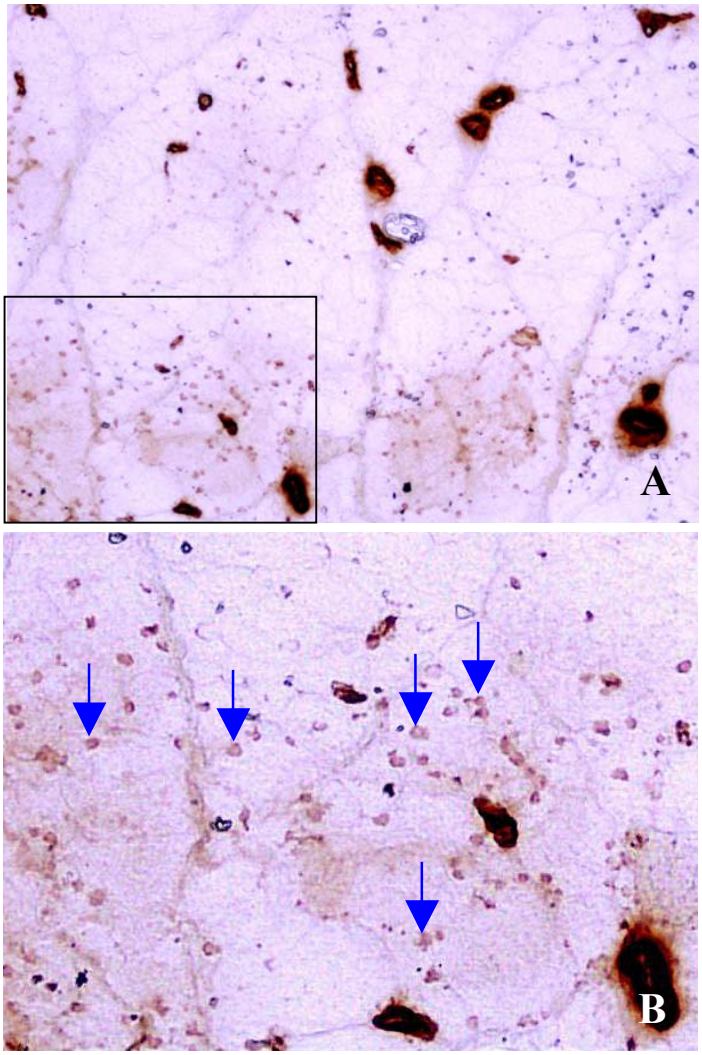

Figure 19. SMA immunohistochemical staining on tissue of Ad-Bicis transduced myoblasts treated group. A. Box region shows numerous SMA expression of newly formed small fine vessels at periphery of muscle fibers at 6 w after transplantation (indicated by blue arrows in B), revealed by DAB substrate (X100). B. Enlarged area of insert in A.

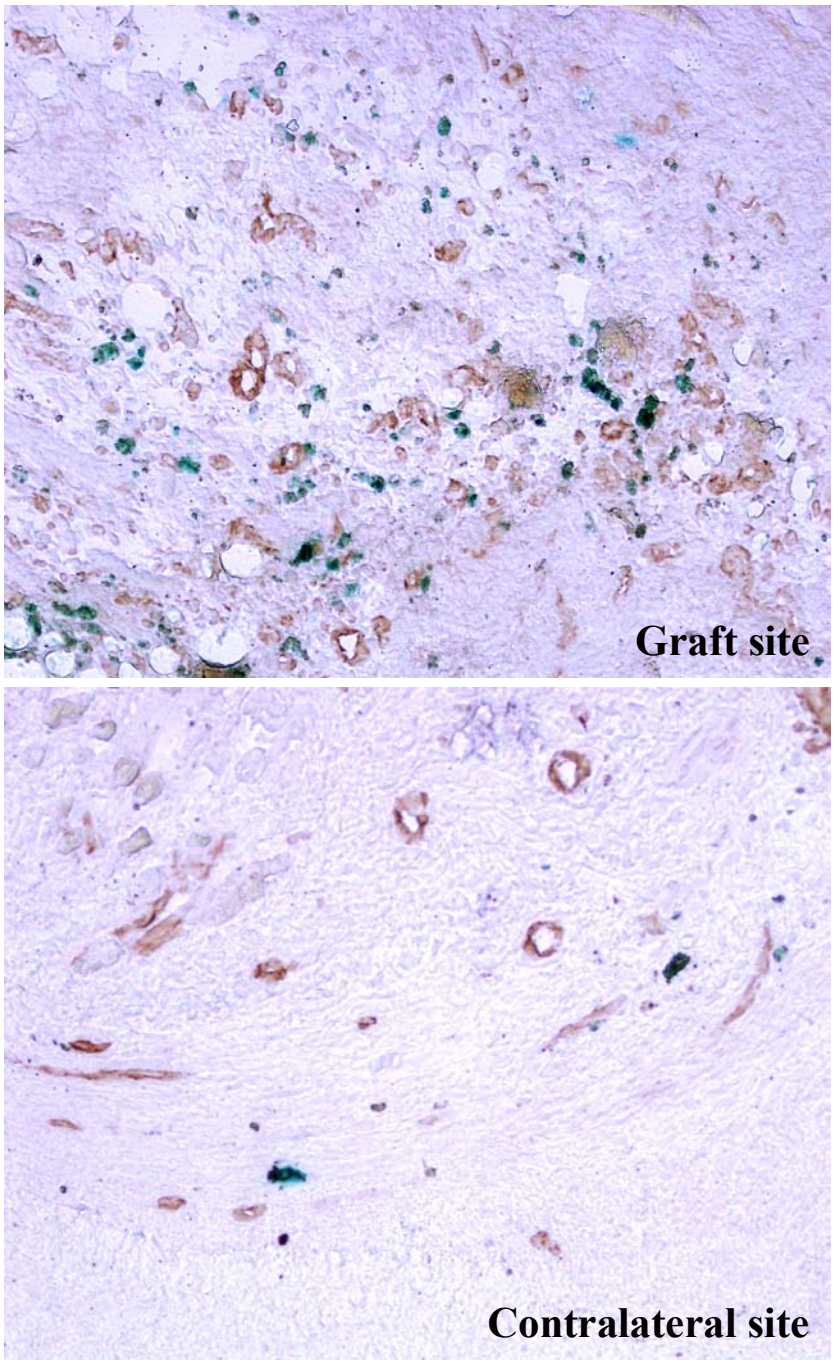

Figure 20. Colocalization of LacZ expressing donor myoblasts and SMA positive vessels in Ad-Bicis transduced myoblasts injected group. Top: Extensive LacZ expression in an ischemic area, 6 w after transplantation, was accompanied by a corresponding increase in SMA positive vessels at site of graft. Bottom: In a contralateral site, minimal LacZ expression was detected, with a corresponding low number of SMA positive vessels. (100x magnifications)

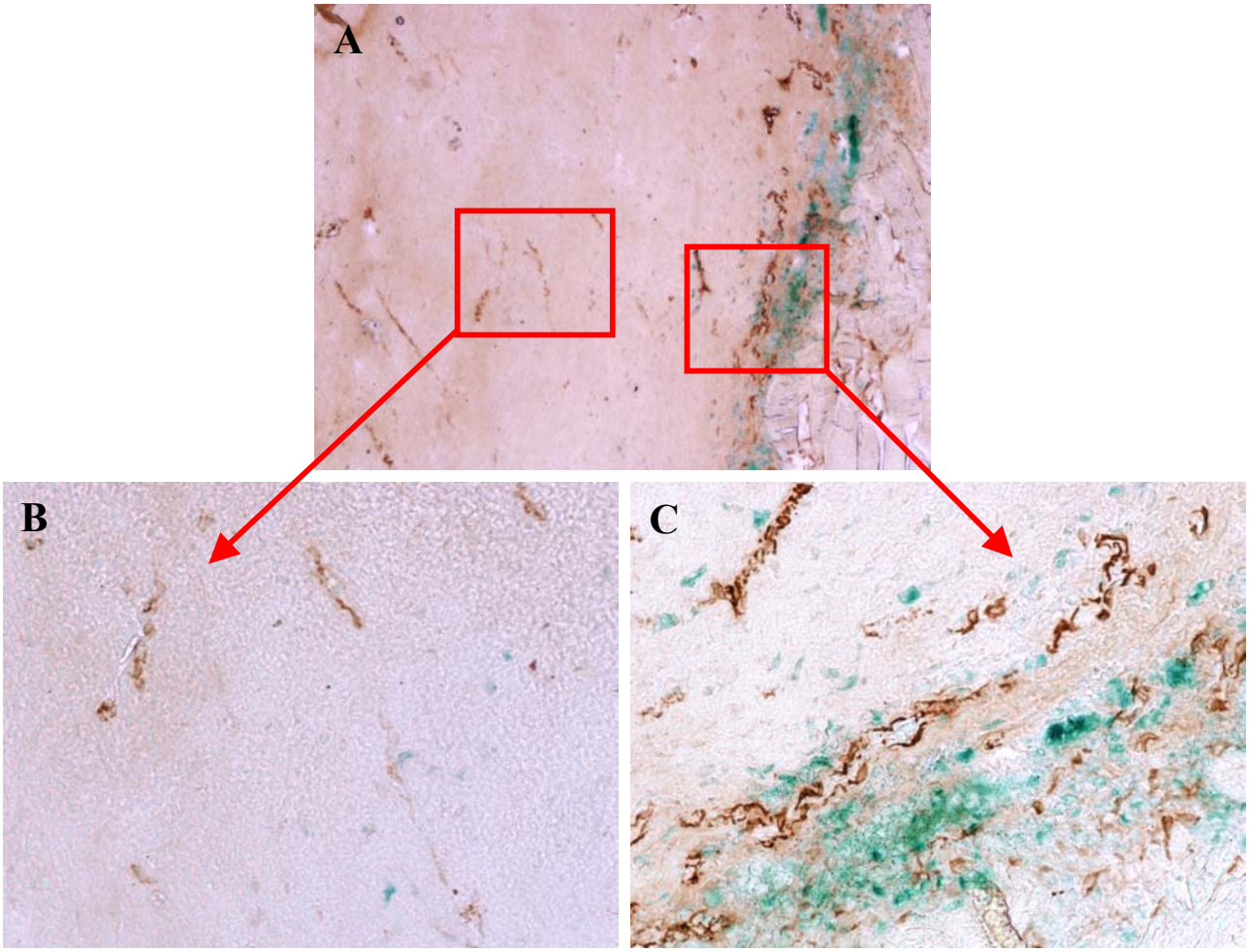

Figure 21. Colocalization of LacZ expressing donor myoblasts and SMA positive vessels in Ad-VEGF transduced myoblasts injected group.
A. Confirmation on proportional degree of expression between LacZ expressing donor myoblasts and SMA positive vessels at 6 weeks after transplantation, taken under low power microscopic field (60x magnification). B. Enlarged area of insert in A - minimal LacZ expression in an ischemic area was accompanied by a corresponding low number of SMA positive vessels (200x magnification) C. Enlarged area of insert in A - extensive LacZ expression was once again accompanied by a corresponding increase in number of SMA positive vessels (200x magnification).

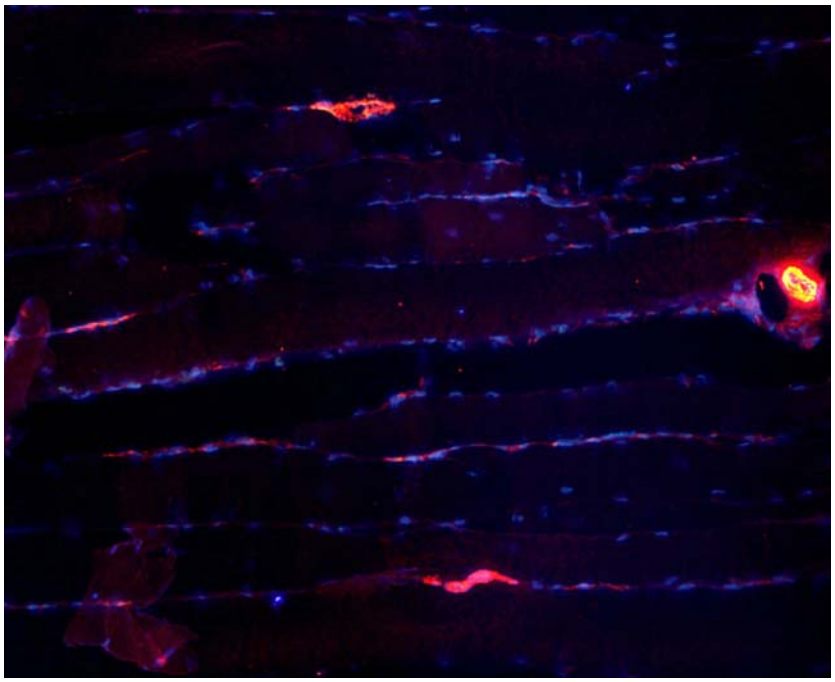

Figure 22. Colocalization of DAPI-labeled donor myoblasts and SMA positive vessels. At site of extensive DAPI-labeled donor myoblasts in a viable muscle region at 8 w after transplanation, increase in TRITC fluorescence of SMA positive vessels were also observed (100x magnification).

This suggested that increased in vessel density was induced by the overexpression of the exogenously introduced angiogenic growth factors from the donor myoblasts.

3.6 *In vivo* secretion of VEGF and expression of myosin heavy chain

Immunohistochemical staining for VEGF on LacZ positive tissue sections obtained from group-5 detected extensive expression of the angiogenic growth factor, both within the vicinity of the transplanted cells or in areas distal to it, in marked contrast to DMEM control group-1 which exhibit only faint VEGF signal (Figure 23). Similarly, myosin heavy chain expression was widespread within the transplanted region, both within the viable muscle fiber region and at sites of ischemic damage (Figure 24).

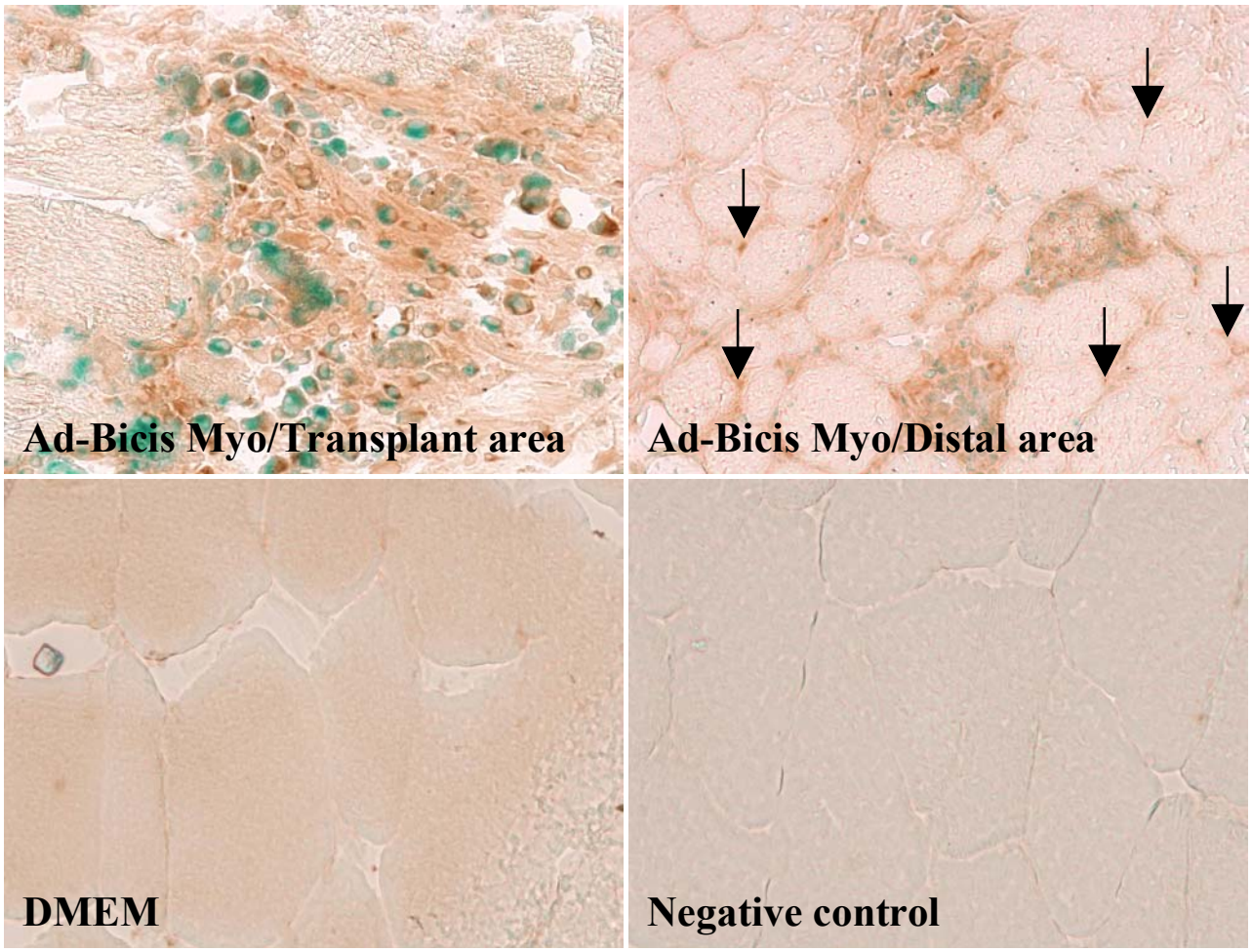

Figure 23. VEGF immunohistochemical staining on LacZ positive tissue sections. Top row: Extensive expression of VEGF demonstrated both within the vicinity of transplanted cells (Left) or areas distal to it (Right, indicated by arrows) of Ad-Bicis transduced myoblasts group at 6 w after transplantation. (200x magnifications) Bottom row: Faint or no VEGF signal from DMEM injected group, 6 w post-treatment (Left) and negative control with primary antibody omitted (Right) used as controls. (300x magnifications)

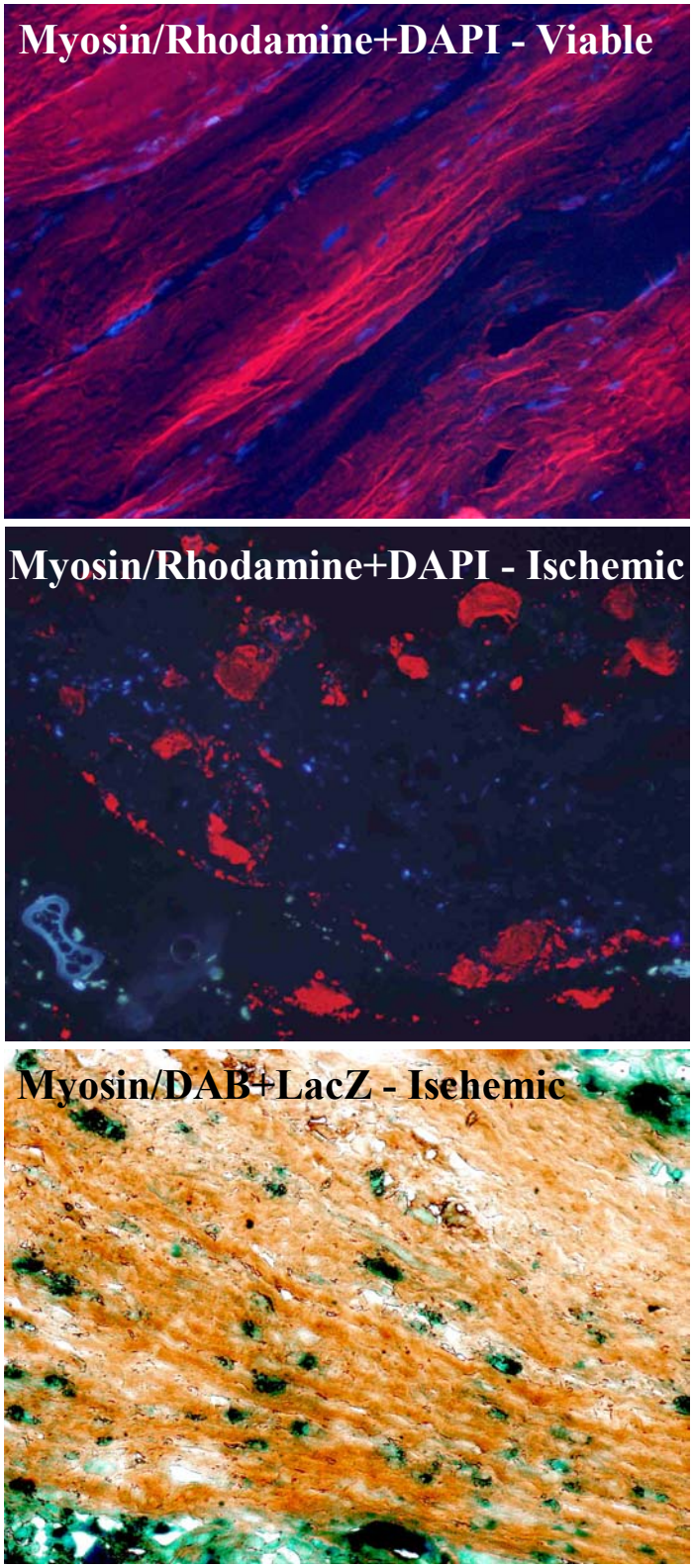

Figure 24. Myosin immunohistochemical staining in transplant area. Myosin heavy chain expression was widespread within the transplant region. Top: Rhodamine fluorescence of myosin heavy chain from viable muscle fibers of a group-4 animal at 6 w after transplantation, colocalized with DAPI-labeled donor myoblasts (150x magnification). Middle: Rhodamine fluorescence of myosin heavy chain in ischemic area also colocalized with DAPI-labeled donor myoblasts in a group-5 animal at 6 w after transplantation (100x magnification). Bottom: Further confirmation from extensive myosin heavy chain expression (revealed by DAB substrate) in region of LacZ expressing donor myoblasts of a group-5 animal at 6 w after transplantation (200x magnification).

4 DISCUSSION

Therapeutic angiogenesis for improvement of peripheral blood flow has been a subject of intense research interest. Both preclinical and clinical studies have been reported in the literature using several treatment modalities for application in PVD (Table 3 and 4). In the former, various angiogenic growth factors have been used in animal models of lower limb ischemia including VEGF, aFGF, bFGF, PDGF, Ang-1 and nitric oxide, together with different routes of administration and various methods to measure the treatment outcome. In the clinical trials, studies ranging from uncontrolled case reports to larger randomized controlled trials, using several different treatment modalities as previously mentioned, have also been reported. Other than the use of different treatment modalities, patient selection criteria and endpoint measurement, as well as different study designs and duration of therapy have been employed. As such, meaningful comparisons on the treatment efficacy are often difficult. There is no doubt that much still needs to be learnt about the optimum treatment modality, dosage and route of administration. However, it is now becoming clear that relying on single growth factor protein or its gene administration is insufficient for the development of a functional vascular system and can cause serious complications (Carmeliet, 2000b; Lee et al., 2000; Celletti et al., 2001; Epstein et al., 2001). These complications are caused mainly by the instability and leakiness of the newly formed vascular networks. There is compelling evidence which suggests that the complex angiogenic process requires more than a single growth factor, involving interplay between various anti- and pro-angiogenic growth factors, and their receptors (Folkman and D'Amore, 1996; Risau, 1997; Carmeliet, 2000a).

In view of these previous reports, more recent studies have focused on the use of combination of factors or their genes. Notably, the synergistic interaction between $VEGF_{165}$ and Ang-1 to produce functional and leakage-resistant neovascularization has been the very reason for their suitability in combination as a promising and safe option for therapeutic angiogenesis. Combining the synergistic effect between $VEGF_{165}$ and Ang-1 together with myoblast therapy for treatment of ischemic limb disease would have the additional advantage of inducing myogenesis on top of the commonly reported angiogenesis in ischemic limbs. The aim of the present study was to deliver both $VEGF_{165}$ and Ang-1 genes in a single administration using a novel bicistronic adenoviral vector that was carried in skeletal myoblasts, to achieve angiomyogenesis to improve overall performance of ischemically damaged hind limb in a rabbit model.

4.1 Skeletal myoblasts purification and culture

In order to achieve these objectives, autologous primary skeletal myoblasts isolated directly from adult rabbit skeletal muscle tissue were used. The reason being that primary skeletal myoblasts more closely approximate myogenesis and do not form tumours *in vivo*, in marked contrast to established myogenic cell lines such as C2C12 (Rando et al., 1994). Established protocols for the isolation and expansion of primary skeletal myoblasts from animal and human skeletal muscles (Rando et al., 1994) now make it possible for skeletal myoblasts to be taken full cycle from the animal to the culture dish and back to the animal. However, the non-myogenic contaminant population of fibroblasts in a primary muscle culture can complicate the results and hence, jeopardize the success of the approach. In fact, presence of these fibroblasts in the transplanted cell

population may persist in the muscle interstitium to produce adverse effects such as increased interstitial connective tissue that will decrease contractility power of the limb. During the present study, this was overcome by enriching the purity of skeletal myoblasts population isolated using modified culture conditions combined with conventional preplating technique. Initial period of skeletal myoblasts culture made use of a patented optimized culture medium (Cell Transplants Singapore Pte. Ltd.). This preferentially supported the growth of skeletal myoblasts relative to fibroblasts. Parallel to this, preplating exploits the differential adhesion characteristics of skeletal myoblasts and fibroblasts (Blanco-Bose et al., 2001). As the latter adhere to tissue culture dishes with greater avidity than skeletal myoblasts, freshly isolated mixed population of cells could be enriched for skeletal myoblasts by preplating the culture on a dish and collecting the cells that have not adhered after 1 h, and transferring them to fresh culture dishes. Apparently, the purity of skeletal myoblast population is enhanced with increased number of preplatings performed (Qu et al., 2000; Qu-Petersen et al., 2002). Some of the skeletal muscle-specific markers such as the transcription factors MyoD, Myf5, myogenin, specific actins or myosins have been used to assess the purity of skeletal myoblasts. However, besides these markers, desmin expression is considered to be more superior as it provides information regarding the ability of skeletal myoblasts to undergo fusion to form multinucleated myotubes. We assessed the purity of skeletal myoblasts in the population of muscle-derived cells by staining the cells for desmin expression. More than 75 to 80 % of the culture skeletal myoblasts stained positive for this myogenic marker. Formation of multinucleated myotubes had been observed in this study when myoblast culture was allowed to grow at high density and in low serum culture conditions. The

high positive desmin expression in the skeletal myoblasts culture gave an early head start as it is also generally accepted that skeletal myoblasts expressing richer levels of desmin show better survival and stability in the host tissue. Skeletal myoblasts of low passage number were used as they are less prone to senescence or transformation. Indeed, it has been demonstrated by others that passage of primary mouse skeletal myoblasts beyond 35 to 40 doublings lead to their spontaneous transformation into immortalized cells which continue to proliferate when implanted *in vivo* (Irintchev et al., 1998; Lu et al., 2001).

These early passage cells were transduced with adenoviral bicistronic vector encoding for both $VEGF_{165}$ and Ang-1. Double fluorescent immunostaining revealed that more than 75 to 80 % of the cells simultaneously expressed both the growth factors. It was noted, however, that the intensity of fluorescent signals for Ang-1 was relatively weaker as compared to $VEGF_{165}$ signals. The difference in expression level of the two growth factors may be attributed to their different position with respect to the promoter region that controlled their expression. As shown in Figure 25, $VEGF_{165}$ gene was located immediately downstream of the murine cytomegalovirus (MCMV) immediate early gene 1 enhancer/promoter whereas that of Ang-1 is much further away, beyond the $VEGF_{165}$ and internal ribosome entry site (IRES) sequence. As such, it was likely that the longer distance between the promoter and Ang-1 decreased its basal expression level, and thus explained in part for the weaker fluorescence emitted by the Ang-1 signal.

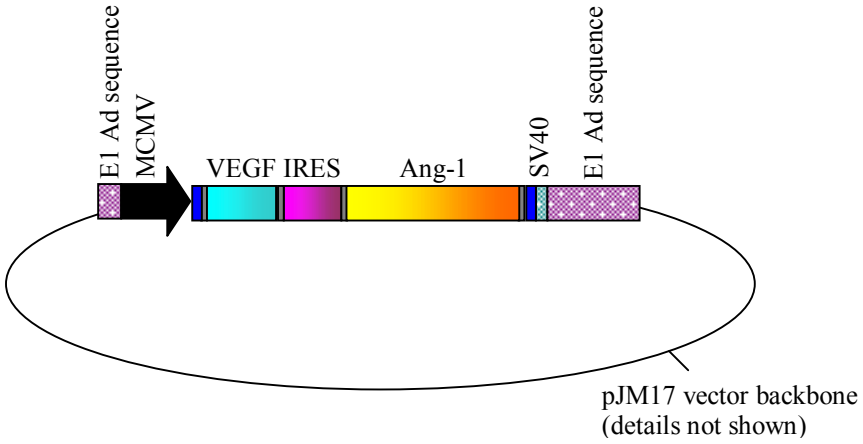

Figure 25. Schematic diagram of recombinant adenovirus expressing $VEGF_{165}$ and Ang-1. (IRES = Internal ribosome entry site; SV 40 = Simian virus 40 polyadenylation signal).

4.2 Animal model development

Ten days prior to implantation, the rabbit model of hind limb ischemia was created by multiple ligatures of the femoral artery. Given the critical importance of such animal models of hind limb ischemia in our understanding of peripheral vascular disease and evaluating therapeutic strategies to improve peripheral circulation, it is imperative that these models closely simulate the human ischemic disease. Unfortunately, the preparation of an animal model of the ischemic limb that is comparable with chronic human ischemia remains a daunting task. The problem is compounded by the remarkable ability in the animals to develop collaterals. It seems that the simple ligation of one or two iliac or femoral arteries could not produce adequate ischemia at rest (Janda et al., 1974; Urbanova et al., 1974; Seifert et al., 1985; Challiss et al., 1986; Hendricks et al., 1990). In this regard, the present study engaged the use of eight-point permanent ligation of the external iliac and femoral arteries of the rabbit to create sufficient ischemia (Figure 3). In

addition, contrary to most other studies who excised a substantial part of the femoral artery (Pu et al., 1993; Asahara et al., 1995; Takeshita et al., 1996; Gowdak et al., 2000a; Masaki et al., 2002; Hershey et al., 2003), we chose only to occlude a short sequence of this vessel that was sufficiently long to avoid simple 'jumping' of collaterals across the ligature (Bauters et al., 1994; Takeshita et al., 1994b, 1995) in order to make it clinically more relevant and with similarity to the natural obliterative disease process. Nonetheless, even with such multiple ligatures, collaterals were still observed at 10 d post-operation (as captured by baseline angiograms). The developing collaterals in the rabbit hind limb after femoral artery occlusion only compensate up to 30 % of the normal maximal flow (Schaper et al., 1976), thus leaving ample room for therapeutic improvement. The rabbit model was chosen as our experimental animal due to its relatively cost effectiveness, easy maintenance, more reliable outcomes of functional studies and less complete formation of collaterals than the dog.

4.3 Skeletal myoblast transplantation and survival of donor cells

Similar to previous studies (Chae et al., 2000), skeletal myoblast transplantation was carried out 10 d after induction of hind limb ischemia to allow for postoperative recovery of animals and to allow the inflammatory response to recede. Inflammation plays an important role in the response to acute ischemia in this model system. Paoni et al. (2002) showed, by histological examination, that immune cell infiltration peaked at 3 d following induction of ischemia and this correlates well with the peak in gene expression of transcripts associated to neutrophils, macrophages, T and B cells. It was also demonstrated that the receptor transcripts for members of most of the major families of

immune cytokines were also induced at this time period. As such, the immediate transplantation of cells into such environment with ongoing inflammatory response following surgical trauma has adverse effects on the survival of the transplanted cells. This is illustrated by Zhang et al. (2001) who observed that transplantation of cardiomyocytes into a vascularized granulation tissue doubles the cell survival compared with injections made into an acutely necrotic cryoinjured myocardium. Besides avoiding the inflammatory response, the 10-d period allowance for skeletal myoblast transplantation was also engaged to allow development of endogenous collateral vessels. Studies done up to 90 d post femoral artery ligation have shown no significant collateral vessel augmentation beyond this 10-d period (Pu et al., 1994) and as such, justify the necessity for therapeutic improvement in vascularization even at this time point.

Following myoblast transplantation, administration of the immunosuppressant cyclosporine and antibiotic cephalexin was carried out. The rationale behind these steps took into consideration previous reports that showed that intramuscular injection of cultured isolated skeletal myoblasts in classical myoblast transfer therapy resulted in massive and rapid necrosis of these donor cells despite their being autologous, with over 90 % dead within the first hour after injection (reviewed in Beauchamp et al., 1999; Hodgetts et al., 2000; Skuk et al., 2000; Smythe et al., 2000c, 2001b). This rapid skeletal myoblasts death appears to be due to exposure to tissue culture conditions (Smythe et al., 2000b) that apparently alter the surface antigenic characterisitics of skeletal myoblasts. Attachment of cells to culture vessel substrate and expansion in growth factors may result in loss or gain of surface receptors (Pouzet et al., 2000). Furthermore, long-term culture of the donor skeletal myoblasts for expansion *in vitro* may allow the animal proteins from

the growth medium to settle down and fix on the suface of the cells and alter their antigenic characteristics. As a consequence, transplantation of these skeletal myoblasts with altered antigenic characterisitics may provoke an acute host immune response *in vivo* which seemed to involve an early nonspecific inflammatory reaction (Grounds 1996; Guerrette et al., 1997a, b) as well as a later, specific cell-mediated immune response (Huard et al., 1994b; Kinoshita et al., 1996a; Guerrette et al., 1997a). To minimize death by immune rejection, transient administration of the immunosuppressant was therefore used. Unfortunately, immunosuppression of the host may affect the behaviour of the transplanted skeletal myoblasts. Apparently, altering the immunologic state of the host could potentially affect the fusion of transplanted myoblasts (Watt, 1982; Hardiman et al., 1993). Furthermore, immunosuppressive agents are feared to have direct toxic effects on host muscle (Le Quintrec et al., 1991). In the light of these reports, immunosuppressant was administered only for a limited time, at the initial phase immediately after cell transplantation.

The success of skeletal myoblast transplantation can generally be determined by a number of measures, including the percentage of skeletal myoblasts that fuse, the number of hybrid myofibers that are formed or the level of transgene expression. Hybrid myofiber formation is critical to efficient systemic delivery of recombinant proteins by the transplanted, genetically engineered myoblasts. Moreover, the ability to detect such hybrid fibers and being able to distinguish them from host fibers that have not been incorporated with donor cells allows for the assessment of the efficacy of myoblast transplantation. In this study, retroviral vectors carrying the nuclear localized *nls*LacZ reporter gene was used to transduce myoblasts as a means of marking these donor cells.

Unlike adenoviral vectors which do not integrate into host chromosome, retroviral vectors allow integration of the LacZ gene into the transduced myoblast genome. As such, progeny of skeletal myoblasts that arise after multiple divisions and propagation, would still retain their LacZ tag without any 'dilutional effect'. Detection of such β-galactosidase activity by X-gal staining in a host myofiber would indicate the survival and engraftment of the donor cells. Long term nuclear expression of LacZ allowed us to study and follow the fate of the donor skeletal myoblasts in the host tissue and provided clear evidence of donor cell survival and formation of hybrid fibers as observed by the intermingling of the green *nls*LacZ-expressing donor nuclei and hematoxylin-stained purple host nuclei (Figure 11). These can be easily distinguished from fibers of the host origin indicated by the presence of only non-LacZ expressing host purple nuclei (Figure 11) (Barr et al., 1991; Dhawan et al., 1991). Moreover, it also allowed assessment of the engraftment, survival and long-term fate of the myoblasts after transplantation. However, since retroviral transduction efficiency of skeletal myoblasts achieved with *nls*LacZ gene was less than 100 %, the cells were addititonally labeled with BrdU and DAPI for post transplant identification.

4.4 Formation of heterokaryons

The donor skeletal myoblasts survived adequately in vivo for ≥ 6 w of our observation as assessed by extensive X-gal staining, and confirmed by immunohistochemical staining for BrdU and DAPI fluorescence. Similar to the previous findings (Rando et al., 1995; Smythe et al., 2000a), the fate of our implanted skeletal myoblasts was either fusion with the host muscle fibers, fusion with regenerating muscle

cells of the host, fusion with other implanted myoblasts, or remaining quiescent as mononucleated cells or death. This observation further supports existing evidence that satellite cells are heterogenous in nature (Schultz et al., 1994; Rantanen et al., 1995; Molnar et al., 1996) with at least two populations – "fusing" and "non-fusing" –isolated from skeletal muscle of various species to date (Baroffio et al., 1996). Although we do not know the extent of cell death among the implanted population, the predominant fate of surviving cells was either fusion to mature host fibers as detected by extensive DAPI-labeled donor nuclei or persistence as β-galactosidase/DAPI labeled mononucleated cells at the site of ischemic region. The possibility of false positive DAPI signals due to DAPI leaking to neighbouring cells or being released upon cell death is very unlikely. Thompson et al. (2003) has shown that injection of lysed DAPI-labeled cells showed no uptake of the dye at 7d post-injection. As for accumulation of donor cells at the site of ischemic region, this is not surprising since it has been demonstrated that at sites of skeletal muscle injury, chemotactic growth factors are produced and these significantly enhanced myoblasts migration to the injured region (Chen and Quinn 1992; Kurek et al. 1996; Bower et al. 1997). Furthermore, there is a strong possibility that fragmentation of the extracellular matrix caused by muscle injury (Morgan et al. 1990, 1993) as in the ischemic area, promotes myoblasts migration compared to the intact matrix in uninjured skeletal muscle.

The resultant hybrid or new myofibers act as platforms for secretion of the exogenously introduced angiogenic growth factors $VEGF_{165}$ and Ang-1. To our knowledge, this is the first study using a bicistronic vector for simultaneous co-expression of the two transgenes $VEGF_{165}$ and Ang-1 in the ischemic limb. Unlike

previous studies which involved two monocistronic vectors to co-administer their combination of growth factor genes (Arsic et al., 2003), utilizing a bicistronic vector provided a less cumbersome delivery option by simplifying the construct design and allowing delivery of the two transgenes in a single administration. Because the vector carries genes that encode for secreted proteins, as opposed to proteins that remain intracellularly, this may yield meaningful biologic outcomes because of the paracrine effects of the secreted gene products. Indeed, *in vivo* secretion of the growth factor was demonstrated in this study by its extensive expression, both within the vicinity of the transplanted cells or in areas distal to it (Figure 23). As such, even when the number of successfully Ad-Bicis transduced skeletal myoblasts implanted had been low, they should still exert a reasonable level of enhanced angiogenesis in the ischemic limb. The secreted angiogenic growth factors give the additional advantage of improved graft survival since they contribute to improved blood supply through both vasodilatation and enhanced angiogenesis. This notion was supported in a recent study by Germani et al. (2003) who demonstrated that VEGF administration to differentiating C2C12 skeletal myoblasts prevented apoptosis, while inhibition of VEGF signaling either with selective VEGF receptor inhibitors or a neutralizing Flk-1 antibody, enhanced cell death approximately by 3.5-fold. Hence, this result points to the supportive role of VEGF in donor skeletal myoblast survival. This is particularly beneficial in the early stage after cell transplantation, when the grafted cells are more prone to the various pathological processes caused by environmental stress such as the ischemic condition of the limb as well as mechanical injury implicated during the cell injection procedure.

4.5 Blood vessel density analysis

The bioactivity of the released angiogenic growth factors was confirmed by increased vascularization detected in the ischemic hind limbs of animals that received Ad-Bicis transduced skeletal myoblast transplantation (group-5) as compared to animals from other groups. This was observed by angiograms that capture radiographically visible collaterals and histological sections immunostained for von Willebrand Factor VIII (vWF) and smooth muscle actin (SMA) to detect the finer capillary networks. Formation of the finer capillary network was an expected outcome of the short term VEGF overexpression, given the very well known stimulating properties of VEGF on endothelial cell proliferation and migration to induce capillary sprouting (Neufield et al., 1999). The combined delivery of VEGF and Ang-1 showed remarkably large proportion of the vessels coated with smooth muscle layer (Figure 18J). These observations were in perfect agreement with the results from previously documented studies showing that Ang-1 was a maturation factor essential for the formation and development of stable, non-leaky vessels (Thurston et al., 2000). We also investigated whether there is a possibility that increase in vessel number was coincidentally due to angiogenesis involved in inflammatory response to granulation tissue, contrary to the underlying treatment received. Tissue sections positive for LacZ expressing or DAPI-labeled donor myoblasts (thus indicating the transplanted region) were counterstained with SMA to visualize functional neovascularization. The result showed that enhanced neovascularization coincides well with regions where there are transplanted cells, be it in tissue area where viable mature muscles were encountered or in areas where ischemically damaged fibers

were observed. This suggests that increased in blood vessel density was genuinely the result of Ad-Bicis transduced myoblasts treatment.

Similar results were observed in group-4 animals as well which displayed a large proportion of vessels positive for the smooth muscle coat. This is intriguing since previous findings have led to the prevalent theory that VEGF administration alone induced formation of merely immature capillaries (Baumgartner et al., 1998; Thurston et al., 1999; Baumgartner, 2000; Thurston et al., 2000; Masaki et al., 2002; Ozawa et al., 2004). Nonetheless, a variety of animal studies have also made it clear that the physiological response to VEGF is complex and can vary depending on the target tissue, mode of delivery and dosage (Bauters et al., 1995; Springer et al., 1998; Lu et al., 2001; Masaki et al., 2002; Arsic et al., 2003). It is reasonable to speculate the difference in our results to myoblast-mediated gene transfer biology and dosage of VEGF delivered. In a study by Ozawa et al. (2004), it was demonstrated that there exists a discrete threshold level in VEGF dosage (~70ng/10^6 cells/day), below which normal, non-leaky, stable capillaries were induced and above which hemangioma growth occurs. Thus, long-term continuous delivery of VEGF, when maintained below a threshold microenvironmental level, can lead to normal angiogenesis without other exogenous growth factors. On the other hand, overexpression of VEGF above this threshold level, without the accompaniment of maturation signals, leads to aberrant angiogenesis. In the present study, the level of VEGF expressed from Ad-VEGF transduced myoblasts could have been below that of this threshold level in which aberrant angiogenesis would have occurred. It must be highlighted that although Ad-VEGF and Ad-Bicis are both derived from human adenovirus type 5, there is a slight difference in the promoter construct of

these two viruses. Whilst expression of VEGF from Ad-VEGF was driven by the relatively weaker human cytomegalovirus (CMV) immediate early gene 1 enhancer/promoter, VEGF and Ang-1 genes in the Ad-Bicis are driven by the stronger murine CMV (MCMV). Indeed this was reflected in the ELISA result that showed higher peak level of VEGF secretion from the Ad-Bicis transduced myoblasts (35.04±1.91 ng/mL - when collected from medium conditioned over 2 d with 10^5 cells seeded; or 175.2 ng/mL/10^6cells/day) compared to that of Ad-VEGF transduced myoblasts whose peak VEGF expression was only 19.52±4.12 ng/mL (when collected from medium conditioned over 2 d with 10^5 cells seeded; or 97.6 ng/mL/10^6cells/day). In this case, the lower level of exogenously introduced VEGF from the Ad-VEGF transduced myoblasts merely acted as an initiator to kick off the angiogenic cascade that recruited other endogenous members involved in the process to eventually form mature blood vessels. However, if in any case the level of VEGF expressed from the myoblast transduced with the Ad-VEGF was above the threshold level, the lack of numerous immature vessels observed could be attributed to myoblast-mediated gene transfer biology. Expressing $VEGF_{165}$ from a myoblast platform possibly involved secretion of other cytokines from the skeletal myoblasts as well which may be responsible for the vessel maturation observed. Indeed, studies have reported the expression of platelet derived growth factor (Sejersen et al. 1986) and epidermal growth factor-like protein (Chen et al. 1995) from myoblasts, both of which play a substantial role in stabilization of newly formed blood vessels (Yancopoulos et al. 2000; Ferrara 2002; Senger et al. 1983; Shing et al. 1984). Besides, recent investigations also support the notion that VEGF-activated endothelial cells may in turn result in the release of other cytokines to

initiate the cascade of events leading to eventual vessel maturation (Yancopoulos et al., 2000). Furthermore, extensive vessel maturation was also observed in animals from groups-2 and -3, as calculated from their maturation index. Taken together, these data supported the suggestion that skeletal myoblasts themselves secrete angiogenic growth factors that contribute to maturation of newly formed blood vessels. Nonetheless, the basal expression of these growth factors from myoblasts are minimal. It is therefore still important that overexpression of exogenously introduced VEGF beyond the threshold level should be accompanied by overexpression of maturation factors such as Ang-1 to circumvent imbalance of angiogenic signals. It would be interesting if an additional control group receiving merely the adenoviral vector carrying VEGF had been included in this study as well. Unless this was done, it would remain difficult to claim the stated hypothesis of cytokine secretion by myoblasts. Nonetheless, a similar study by Hershey et al. (2003) demonstrated that intramuscular injection of Ad-$VEGF_{165}$ into the ischemic limb of rabbit model led to an increase in capillary density but not an increase to the growth and development of those larger conduit vessels. In fact, Ad-$VEGF_{165}$ administered at high dose even caused hind limb edema. This study, in comparison to ours, therefore provides some insights to differences between myoblast-mediated gene transfer and direct viral vector gene transfer biology.

More importantly though, despite displaying similar maturation index across all groups, group-5 still sustained the highest blood vessel density count. This was in perfect agreement to previous studies reporting higher capillary density in rabbit ischemic hind limbs that received combined VEGF and Ang-1 administration as compared to those receiving either only VEGF or Ang-1 alone (Chae et al., 2000; Arsic et al., 2003; Shyu et

al., 2003a). The results of this study underlined the notion that synergistic interaction between $VEGF_{165}$, Ang-1 and cytokines released from skeletal myoblasts provided the optimal cocktail of angiogenic growth factors essential for enhanced functional neovascularization. Though the physiological consequences of such increased vessel density were not assessed in this study, it seemed plausible that the increased microvascular capacity should, for a similar level of blood flow, allow better oxygen extraction by the ischemic muscles and consequently, better muscle performance.

Angiographic evidence of improved arteriogenesis in group-5 further supported the enhanced neovascularization detected by immunohistochemistry for vWF and SMA. Although there was no statistically significant difference among groups-3, -4, and -5, more collaterals were formed in the order of group-5 > group-4 > group-3. Moreover, angiographic score comparison between group-5 versus the groups-2 and -3 revealed significant improvement in group-5. This result is of pivotal interest since from a therapeutic point of view, it is the growth and development of the larger conduit vessels (as captured by angiography) that is most likely to impart functional improvement by reducing vascular resistance imposed by the occluded vessel in an ischemic area through restoration of blood flow to the underperfused areas at risk for necrosis or loss of function. The angiographic score result was inconsistent with capillary density. Wherelse capillary density clearly showed a marked difference between group-5 as compared to all the other groups, such a difference was not as significant when reflected at the angiographic level. This slight discrepancy may be attributed to the different sensitivity and specificity of the two techniques used. Whilst histological study on vascular density allows for detection of small vessels such as capillaries and arterioles of less than 40 μm

in diameter, the resolution of the angiographic apparatus is less sensitive and precludes the visualization of vessels less than 200 μm in diameter (Mori et al., 1994; Takeshita et al., 1997). Perhaps, the size of arterioles that reflect the significant difference between groups-5, -4, and -3 were too small to permit angiographic detection of the newly formed vascular network. However, it must also be emphasized that we only measured arteriogenesis, but not signs of improvement to the previously underperfused tissue. Studies using biochemical markers of impaired blood supply will further elucidate whether the lower leg was still ischemic or has truly improved in their blood supply.

The use of adenovirus in this study is justified by its transient expression of the transgenes. Unlike retroviral vector that is stably integrated into the host genome, adenovirus remains outside the genome as an episome, and as such result in progressive loss of the transgene expression. This is desirable as long-term stable expression of the angiogenic growth factor genes often results in deleterious effects such as hemangiomas (Springer et al., 1998; Lee et al., 2000). The transient expression of VEGF secreted from our Ad-Bicis transduced myoblasts was confirmed by ELISA and it clearly showed a single peak expression at 6 d post transduction, thereafter dwindling progressively to an insignificant value by 28 d post transduction. However, despite *in vitro* data showing cessation of VEGF overexpression beyond 28 d, we still observed extensive *in vivo* VEGF expression in Ad-Bicis transduced myoblasts transplanted group at 6 w after transplantation (Figure 23). This discrepancy could be attributed to the fact that the *in vivo* expression of these angiogenic growth factors are complex and can vary depending on the target tissue, mode of delivery and dosage (Bauters et al., 1995; Springer et al., 1998; Lu et al., 2001; Masaki et al., 2002; Arsic et al., 2003). For instance, the

proliferative rate of myoblasts transduced with the Ad-Bicis within the three-dimensional, compact environment of a limb tissue might be slower than when these cells were allowed to be propagated on the two-dimensional growth surface of culture dishes that apparently have much more growth space for cell expansion. As such, in comparison to those cells grown *in vitro*, the slower-cycling nature of these transplanted cells would lead to their Ad episome being retained for a longer period of time before undergoing 'dilutional effect' and thus progressive loss of the transgene expression. This may explain why extensive *in vivo* VEGF expression in Ad-Bicis transduced myoblasts transplanted group was observed even at 6 w after transplantation.

Despite eventual progressive loss of the transgene expression in vivo, numerous mature blood vessels in the Ad-Bicis transduced myoblasts group was demonstrated even up to 8 w post-transplantation. Continuous VEGF supplementation is instrumental in preventing the apoptosis of vascular endothelial cells in immature blood vessels to maintain their viability (Alon et al. 1995). Upon maturation though, VEGF is no longer required for their survival (Benjamin et al. 1999). As such, this accounts for the relatively long-term maintenance of the mature blood vessels observed in the Ad-Bicis transduced myoblasts group. Hence, this is in line with the long term requirement for treatment of true severe chronic limb ischemia patients.

4.6 Skeletal myoblasts as carriers of the angiogenic genes

Another important difference in this study compared to others lies in the use of skeletal myoblasts as carriers of the bicistronic adenoviral vector. To date, the use of direct viral vector based gene delivery has remained questionable due to its serious

immunologic concerns. Adverse reactions from incomplete inactivation of the viral replication machinery may lead to life-threatening systemic inflammatory-like reactions (Leiden, 2000; Verma, 2000). Furthermore, it has been reported that administration of adenoviral vectors immediately after induction of acute ischemia in rat hind limbs caused inflammation and subsequent limb gangrene that seemed to be aggravated by the adenovirus capsid protein (Brevetti et al., 2001). As such, this justifies our rationale for using myoblast-mediated gene delivery as a safe and promising protocol for gene therapy. Furthermore, the Ad-Bicis transduced myoblasts can be fully characterized *in vitro* before transplantation to ensure that the expression of the growth factors are of the correct size and function at pharmacologically useful levels. On the contrary, direct viral vector mediated gene transfer is often limited by the variability in the number of endogenous cells that take up and express the foreign gene. Besides, utilizing skeletal myoblasts as carriers of the bicistronic vector has the additional advantage of contributing to myogenesis for repair and repopulating of the traumatized muscle due to the ischemic damage. As documented by others and confirmed in this study (Figure 11-13), intramuscularly injected myoblasts are capable of fusing with pre-exisitng muscle fibers as well as with one another. In the resulting heterokaryotic muscle fibers, the donor nuclei are expected to supplement the lost genes in the host nuclei due to the ischemic injury. Moreover, being precursors of skeletal muscle, this fusion process should result in augmentation of the number of contractile elements in the damaged host muscle, possibly even resurrecting some surviving host cells in the necrotic region. Indeed, we observed extensive expression of skeletal muscle myosin heavy chain within the vicinity of LacZ-expressing or DAPI-labeled donor skeletal myoblasts (Figure 24), suggestive of possible

neomyogenesis at the site of the cell graft from the donor skeletal myoblasts-mediated induction of myoblasts differentiation into skeletal muscle fiber to repopulate the ischemic area where muscle fiber disintegration occurred.

4.7 Conclusions and future directions

In conclusion, *ex vivo* angiogenic growth factor gene delivery by myoblast-mediated transfer offers a potent option for therapeutic angiogenesis in limb ischemia. Our study gave evidence that concerted action amongst VEGF, Ang-1 and possibly cytokines released from myoblasts is capable of inducing enhanced functional neovascularization. Combined with the therapeutic myogenesis effect contributed by the myoblasts themselves, angiomyogenesis utilizing the duo combination of myoblast and angiogenic growth factor genes is an emerging therapeutic target for patients suffering from PVD.

It is therefore of high priority to establish the safety of this treatment strategy. Future direction should take into consideration employing larger numbers of animals as well as using larger animal models such as porcines that are more physiologically similar to the human body to ascertain its safety issue. In addition, in line with treatment of true severe chronic limb ischemia patients, the treatment period should be extended longer beyond the existing eight-week period. More interestingly, a different approach utilizing lipofection to deliver the angiogenic growth factor genes to the myoblasts rather than the current adenoviral mediated transduction could be employed to achieve an improved foolproof safety of the procedure.

Given the availability of resources, it is also of considerable interest that more functional studies to evaluate signs of improvement to the previously underperfused tissue be performed. This may include measuring the regional blood flow to limb muscles for any indication of improved perfusion in the ischemic region either by microspheres administration, SPECT-sestamibi scan or Doppler guidewire. Use of the more reliable transcutaneous oxygen and carbon dioxide pressure foot monitoring as a metabolic test of actual tissue perfusion could also be used to discriminate the severity of the limb ischemia before and after treatment. Indeed, this will further elucidate whether the lower leg is still ischemic or has truly improved in their blood supply.

The increase in microvascular capacity should, for a similar level of blood flow, allow better oxygen extraction by the ischemic muscles and consequently lead to better muscle performance; not to mention the additional therapeutic myogenesis benefit from the myoblast transplantation itself. As such, besides assessing response to therapeutic angiogenesis, future study should also take into account of evaluating improvement to the muscle. One such study to assess improvement of the bioenergetic reserve of the muscle is the use of ^{31}P nuclear magnetic resonance spectroscopy. Maximum twitch tension and tetanic tension should also be measured to assess force generation while fatigue index can be used as an indicator to assess the number of limbs that recovered.

Despite the novelty of therapeutic angiomyogenesis in treatment of limb ishcemia, cautious optimism is warranted as the field progresses from benchwork to bedside. Our data may provide guidance for the ongoing clinical trials of angiogenic growth factors for the treatment of ischemic limb disease.

BIBLIOGRAPHY

Albers M, Fratezi AC, DeLuccia N (1992) Assessment of quality of life of patients with severe ischemia as a result of infrainguinal arterial occlusive disease. J Vasc Surg 16:54-59

Al-Khaldi A, Al-Sabti H, Galipeau J, Lachapelle K (2003) Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. Ann Thorac Surg 75:204-209

Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1: 1024–1028

Arsic N, Zentilin L, Zacchigna S, Santoro D, Stanta G, Salvi A, Sinagra G, Giacca M (2003) Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. Molecular Therapy 7:450-459

Asahara T, Bauters C, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM (1995) Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 92:II365-II371

Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P (1992) Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. J Vasc Surg 16:181-191

Baffour R, Garb JL, Kaufman J, Berman J, Rhee SW, Norris MA, Friedmann P (2000) Angiogenic therapy for the chronically ischemic lower limb in a rabbit model. J Surg Res 93:219-29

Baroffio A, Hamann M, Bernheim L, Bochaton-Piallat ML, Gabbiani G, Bader CR (1996) Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. Differentiation 60:47-57

Barr E, Leiden JM (1991) Systemic delivery of recombinant proteins by genetically modified myoblasts. Science 254:1507-1509

Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 97:1114-1123

Baumgartner I (2000) Therapeutic angiogenesis: theoretic problems using vascular endothelial growth factor. Curr Cardiol Rep 2:24-28

Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM (1994) Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. Am J Physiol 267:H1263-1271

Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, Ferrara N, Symes JF, Isner JM (1995) Recovery of distrurbed endothelium–dependent flow in the collateral-perfused rabbit ischemic hind limb after administration of vascular endothelial growth factor. Circulation 91:2802-2809

Beauchamp JR, Morgan JE, Pagel CN, Partridge TA (1994) Quantitative studies of the efficacy of myoblast transplantation. Muscle Nerve 18:S261

Beauchamp JR, Morgan JE, Pagel CN, Partridge TA (1999) Dynamics of myoblast transplantation reveals a discrete minority of precursors with stem cell-like properties as the myogenic source. J Cell Biol 144:1113-1122

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103:159 –165

Bett AJ, Haddara W, Prevec L, Graham FL (1994) An efficient and flexible system for construction of adenovirus vectors with insertion or deletion in early regions 1 and 3. Proc Natl Acad Sci USA 91:8802-8806

Bischoff R (1994) The satellite cell and muscle regeneration. In: Myogenesis (Engel AG and Franszini-Armstrong C, Eds) McGraw-Hill New York Vol 2 pp 97-118

Blanco-Bose WE, Yao C-C, Kramer RH, Blau HM (2001) Purification of mouse primary myoblasts based on α7 integrin expression. Exp Cell Res 265:212–220

Blau HM, Rossi FM (1999) Tet B or not Tet B: advances in tetracycline-inducible gene expression. Proc Natl Acad Sci USA 96:797-799

Bohl D, Naffakh N, Heard JM (1997) Long-term control of erythropoietin secretion by doxycycline in mice transplanted with engineered primary myoblasts. Nat Med 3:299-305

Bower JJ, White JD, Kurek JB, Muldoon CM, Austin L (1997) The role of growth factors in myoblast transfer therapy. Basic Appl Myol 7:177-186

Brevetti LS, Sarkar R, Chang DS, Ma M, Paek R, Messina LM (2001) Administration of adenoviral vectors induces gangrene in acutely ischemic rat hindlimbs: role of capsid protein-induced inflammation. J Vasc Surg 34:489-96

Brevetti LS, Chang DS, Tang GL, Sarkar R, Messina LM (2003) Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. J Vas Surg 38:820-826

Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y (2003) Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nature Med 9:604-613

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435-439

Carmeliet P (1999a) Basic concepts of (myocardial) angiogenesis: role of vascular endothelial growth factor and angiopoietin. Curr Interv Cardiol Rep 1:322-335

Carmeliet P, Collen D (1999b) Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. Curr Top Microbiol Immunol 237:133-158

Carmeliet P (2000a) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389-395

Carmeliet P (2000b) VEGF gene therapy:stimulating angiogenesis or angioma-genesis? Nat Med 6:1102-1103

Caron NJ, Asselin I, Morel G, Tremblay JP (1999) Increased myogenic potential and fusion of matrilysin-expressing myoblasts transplanted in mice. Cell Transplant 8:465-476

Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD (2001) Vascular endothelial growth factor enhances artherosclerotic plaque progression. Nat Med 7:425-429

Coffman JD (1991) Intermittent claudication-be conservative. N Engl J Med;325:577-578

Chae JK, Kim J, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY (2000) Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol 20:2573-2578

Challiss RAJ, Hayes DJ, Petty RFH, Radda GK (1986) An investigation of arterial insufficiency in rat hindlimb: a combined 31P-n.m.r. and blood flow study. Biochem J 236:461-467

Chang DS, Su H, Tang GL, Brevetti LS, Sarkar R, Wang R, Kan YW, Messina LM (2003) Adeno-associated viral vector-mediated gene transfer of VEGF normalizes

skeletal muscle oxygen tension and induces arteriogenesis in ischemic rat hindlimb. Mol Ther 7:44-51

Chen G, Quinn LS (1992) Partial characterization of sleletal myoblast mitogens in mouse crushed muscle extract. J Cell Physiol 153:563-574

Chen X, Raab G, Deutsch U, Zhang J, Ezzell RM, Klagsbrun M (1995) Induction of heparin-binding EGF-like growth factor expression during myogenesis. Activation of the gene by MyoD and localization of the transmembrane form of the protein on the myotube surface. J Biol Chem 270:18285-18294

Cherng JM, Lin CM, Lin CL, Huang SM, Chang HL, Lee CC, Chiang LC, Chang PY (2000) Effects of VEGF121 and/or VEGF165 gene transfection on collateral circulation development. J Formos Med Assoc 99:603-1

Chleboun JO, Martins RN, Mitchell CA, Chirila TV (1992) bFGF enhances the development of the collateral circulation after acute arterial occlusion. Biochem Biophys Res Commun 185:510-516

Chleboun JO, Martins RN (1994) The development and enhancement of the collateral circulation in an animal model of lower limb ischemia. Aust NZ J Surg 64:202-207

Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J (2000) Generalized potential of adult neural stem cells. Science 288:1660-1663

Cooper LT Jr, Hiatt WR, Creager MA, Regensteiner JG, Casscells W, Isner JM, Cooke JP, Hirsch AT (2001) Proteinuria in a placebo-controlled study of bFGF for intermittent claudication. Vasc Med 6:235–239

Crystal RG (1997) Phase I study of direct administration of a replication deficient adenovirus vector containing E coli cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the pro-drug 5-fluorocytosine. Hum Gene Ther 8:985 – 1001

Dai Y, Roman M, Naviaux RK, Verma IM (1992) Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation in vivo. Proc Natl Acad Sci USA 89:10892-10895

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD (1996) Isolation of angiopoietin-1, a ligand for the Tie2 receptor, by secretion-trap expression cloning. Cell 87:1161-1169

De Angelis L, Berghella L, Coletta M, Lattanzi L, Zanchi M, Cusella-De Angelis MG, Ponzetto C, Cossu G (1999) Skeletal myogenic progenitors originating from embryonic dorsal aorta co-express endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. J Cell Biol 147**:**869-878

Dhawan J, Pan LC, Pavlath GK, Travis MA, Lanctot AM, Blau HM (1991) Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. Science 254:1509-1512

Dvorak HF, Nagy JA, Feng D, Brown LF, Dvoral AM (1999) Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr. Top Microbiol Immunol 237:97-132

El Fahime E, Torrente Y, Caron N, Bresolin M, Tremblay JP (2000) In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity. Exp Cell Res 258:279-287

Epstein SE, Kornowski R, Fuchs S, Dvorak HF (2001) Angiogenesis therapy: amidst the hype, the neglected potential for serious side effects. Circulation 104:115-119

Eriksson U, Alitalo K (1999) Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. Curr Top Microbiol Immunol 237:41-57

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84:7413-7417

Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851-858

Ferrara, N, Leung DW, Cachianes G, Winer J, Henzel WJ (1991) Purification and cloning of vascular endothelial growth factor secreted by folliculo-stellate cells. Methods Enzymol 198:391-405

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439-442

Ferrara N (1999) Vascular endothelial growth factor: Molecular and biological aspects. Curr Top Microbiol Immunol 237:1-30

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795-803

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528-1530

Folkman J, Klagsburn M (1987) Angiogenic factors. Science 235:442-447

Folkman J, D'Amore PA (1996) Blood vessel formation: what is its molecular basis? Cell 87:1153-1155

Fong G-H, Rossant J, Gersenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66-70

Fox JC, Swain JL (1996) Angiogenic gene therapy. A leg to stand on? Circulation 94:3065-3066

Gale NW, Yancopoulos GD (1999) Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev 13:1055-1066

Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M, Mora M, De Angelis MG, Fiocco R, Cossu G, Vescovi AL (2000) Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci 3:986-991

Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N (1999a) VEGF is required for growth and survival in neonatal mice. Development 126:1149-1159

Gerber HP, Vu TH, Ryan AM, Kowalski J, Wreb Z, Ferrara N (1999b) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nature Med 5:623-628

Germani A, Di Carlo A, Mangoni A, Straino S, Giacinti C, Turrini P, Biglioli P, Capogrossi MC (2003) Vascular endothelial growth factor modulates skeletal myoblast function. Am J Pathol 163:1417-1428

Gilgenkrantz H, Duboc D, Juillard V, Couton D, Pavirani A, Guillet JG, Briand P, Kahn A (1995) Transient expression of genes transferred in vivo into heart using first-generation adenoviral vectors: role of the immune response. Hum Gene Ther 6:1265–1274

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG (1998) Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 78:1385-1394

Goldman CK, Soroceanu L, Smith N, Gillespie GY, Shaw W, Burgess S, Bilbao G, Curiel DT (1997) In vitro and in vivo gene delivery mediated by a synthetic polycationic amino polymer. Nature Biotechnol 15:462-466

Gowdak LH, Poliakova L, Li Z, Grove R, Lakatta EG, Talan M (2000a) Induction of angiogenesis by cationic lipid-mediated VEGF165 gene transfer in the rabbit ischemic hindlimb model. J Vasc Surg 32:343-352

Gowdak LH, Poliakova L, Wang X, Kovesdi I, Fishbein KW, Zacheo A, Palumbo R, Straino S, Emanueli C, Marrocco-Trischitta M, Lakatta EG, Anversa P, Spencer RG, Talan M, Capogrossi MC (2000b) Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. Circulation 102:565-571

Grounds MD (1996) Commentary on the present state of knowledge for myoblast transfer therapy. Cell Transplant 3:431-433

Guerrette B, Asselin I, Skuk D, Entman M, Tremblay JP (1997a) Control of inflammatory damage by anti-LFA-1: increased success of myoblast transplantation. Cell Transplant 6:101-107

Guerrette B, Skuk D, Celestin F, Huard C, Tardif F, Asselin I, Roy B, Goulet M, Roy R, Entman M, Tremblay JP (1997b) Prevention by anti-LFA-1 on acute myoblast death following transplantation. J Immunol 159:2522-2531

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390-394

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, Le Deist F, Wulffraat N, Mcintyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Eng J Med 348:255-256

Haider Kh H, Ye L, Jiang S, Ge R, Law PK, Chua T, Wong P, Sim EK (2004) Angiomyogenesis for cardiac repair using human myoblasts as carriers of human vascular endothelial growth factor. J Mol Med Jun 3 [Epub ahead of print]

Halperin JL (2002) Evaluation of patients with peripheral vascular disease. Thromb Res 106:V303-311

Hamamori Y, Samal B, Tian J, Kedes L (1994) Persistent erythropoiesis by myolablast transfer of erythropoietin cDNA. Hum Gene Ther 5:1349-1356

Hamamori Y, Samal B, Tian J, Kedes L (1995) Myoblast transfer of human erythropoietin gene in a mouse model of renal failure. J Clin Invest 95:1808-1813

Hamano K, Li TS, Kobayashi T, Tanaka N, Kobayashi S, Matsuzaki M, Esato K (2001) The induction of angiogenesis by the implantation of autologous bone marrow cells: a novel and simple therapeutic method. Surgery 130:44-54

Hardiman O, Sklar RM, Brown RH Jr (1993) Direct effects of cyclosporin A and cyclophosphamide on differentiation of normal human myoblasts in culture. Neurology 43:1432-1434

Hayashi S, Morishita R, Nakamura S, Yamamoto K, Moriguchi A, Nagano T, Taiji M, Noguchi H, Matsumoto K, Nakamura T, Higaki J, Ogihara T (1999) Potential role of hepatocyte growth factor, a novel angiogenic growth factor, in peripheral arterial disease: down regulation of HGF in response to hypoxia in vascular cells. Circulation 100:II301-II308

Hellstrom M, Kaln M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth cells and pericytes during embryonic blood vessel formation in the mouse. Development 126:3047-3055

Hendricks DL, Pevec WC, Shestak KC, Rosenthal MC, Webster MW, Steed DL (1990) A model of persistent partial hindlimb ischemia in the rabbit. J Surg Res 49:453-457

Hershey JC, Baskin EP, Corcoran HA, Bett A, Dougherty NM, Gilberto DB, Mao X, Thomas KA, Cook JJ (2003) Vascular endothelial growth factor stimulates angiogenesis without improving collateral blood flow following hindlimb ischemia in rabbits. Heart Vessels 18:142-149

Hirakata K, Li TS, Nishida M, Ito H, Matsuzaki M, Kasaoka S, Hamano K (2003) Autologous bone marrow cell implantation as therapeutic angiogenesis for ischemic hindlimb in diabetic rat model. Am J Physiol Heart Circ Physiol 284:H66-70

Hirschi KK, Rohovsky SA, Beck, LH, Smith SR, D'Amore PA (1999) Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 84:298-305

Hodgetts SI, Beilharz MW, Scalzo T, Grounds MD (2000) Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ or NK1.1+ cells. Cell Transplant 9:489-502

Hopkins SP, Bilgrin JP, Sims RL, Bowman B, Donovan DL, Schmidt SP (1998) Controlled delivery of vascular endothelial growth factor promotes neovascularization and maintains limb function in a rabbit model of ischemia. J Vasc Surg 275:886-894

Huang YQ, Li JJ, Karpatkin S (2000) Identification of a family of alternatively spliced mRNA species of angiopoietin-1. Blood 95:1993-1999

Huard J, Ascadi G, Jani A, Massie B, Karpati G (1994a) Gene transfer into skeletal muscles by isogenic myoblasts. Hum Gene Ther 5:949-958

Huard J, Roy R, Guerrette B, Verreault S, Tremblay G, Tremblay JP (1994b) Human myoblast transplantation in immunodeficient and immunosuppressed mice: evidence of rejection. Muscle Nerve 17:224-234

Iba O, Matsubara H, Nozawa Y, Fujiyama S, Amano K, Mori Y, Kojima H, Iwasaka T (2002) Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs. Circulation 106:2019-2025

Ikenaga S, Hamano K, Nishida M, Kobayashi T, Li TS, Kobayashi S, Matsuzaki M, Zempo N, Esato K (2001) Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model. J Surg Res 96:277-283

Irintchev A, Rosenblatt JD, Cullen MJ, Zweyer M, Wernig A (1998) Ectopic skeletal muscles derived from myoblasts implanted under the skin. J Cell Sci 111:3287-3297

Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF (1996a) Clinical evidence of angiogenesis after arterial gene transfer of phVEGF 165 in patient with ischemic limb. Lancet 348:370–374

Isner JM, Walsh K, Symes J, Pieczek A, Takeshita S, Lowry J, Rosenfield K, Weir L, Brogi E, Jurayj D (1996b) Arterial gene transfer for therapeutic angiogenesis in patients with peripheral artery disease. Hum Gene Ther 7:959-88

Isner JM (1998) Vascular endothelial growth factor: gene therapy and therapeutic angiogenesis. Am J Cardiol 82:63S-64S

Ito H, Hallauer PL, Hastings KE, Tremblay JP (1998) Prior culture with concanavalin A increases intramuscular migration of transplanted myoblast. Muscle Nerve 21:291-297

Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T (2002) Endothelial progenitor cell VEGF gene transfer for vascular regeneration. Circulation 105:732–738

Iwama A, Hamaguchi I, Hashiyama M, Mutayama Y, Yasunaga K, Suda T (1993) Molecular cloning and characterization of mouse Tie and Tek receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochem Biophys Res Commun 195:301-309

Jackson RJ, Howell MT, Kaminski A (1990) The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem Sci 15:477-483

Janda J, Linhart J, Kasalicky J (1974) Experimental chronic ischemia of the skeletal muscle in the rat. Physiol Bohemoslov 23:521-526

Kim I, Moon SO, Koh KN, Kim H, Uhrn CS, Kwak HJ, Kim NG, Koh GY (1999) Molecular cloning, expression, and characterization of angiopoietin-related protein. J Biol Chem 274:26523-26528

Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, Ahn BC, Koh GY (2000a) Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. Circ Res 86:952-959

Kim I, Kim HG, So JN, Kim JH, Kwak HJ, Koh GY (2000b) Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Circ Res 86:24-29

Kinoshita I, Vilquin JT, Guerette BG, Asselin I, Tremblay JP (1994) Very efficient myoblast transplantation in mice under FK506 immunosuppression. Muscle Nerve 17:1407-1415

Kinoshita I, Vilquin JT, Tremblay JP (1995) Pretreatment of myoblast cultures with basic fibroblast growth factor increases the efficacy of their transplantation in mdx mice. Muscle Nerve 18:834-841

Kinoshita I, Roy R, Dugre FJ, Gravel C, Roy B, Goulet M, Asselin I, Tremblay JP (1996a) Myoblast transplantation in monkeys: control of immune response by FK506. J Neuropathol Exp Neurol 55:687-697

Kinoshita I, Vilquin JT, Roy T, Tremblay JP (1996b) Successive injections in mdx mice of myoblasts grown with bFGF. Neuromuscul Disord 6:187-193

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W (1998) Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr Biol 8:529-532

Kondoh K, Koyama H, Miyata T, Takato T, Hamada H, Shigematsu H (2004) Conduction performance of collateral vessels induced by vascular endothelial growth factor or basic fibroblast growth factor. Cardiovasc Res 61:132-42

Korhonen J, Polvi A, Partanen J, Alitalo K (1994) The mouse Tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene 9:395-403

Kozak M (1989) The scanning model for translation: an update. J Cell Biol 108:229-241

Kringstein AM, Rossi FM, Hofmann A, Blau HM (1998) Graded transcriptional response to different concentration of a single transactivator. Proc Natl Acad Sci USA 95:13670-13675

Kroll J, Waltenberger J (1998) VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). Biochem Biophys Res Commun 252:743–746

Kurek JB, Nouri S, Kannourakis G, Murphy M, Austin L (1996) Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle. Muscle Nerve 19:1291-1301

Kyriakides ZS, Petinakis P, Kaklamanis L, Sbarouni E, Karayannakos P, Iliopoulos D, Dontas I, Kremastinos DT (2001) Intramuscular administration of estrogen may promote angiogenesis and perfusion in a rabbit model of chronic limb ischemia. Cardiovasc Res 49:626-633

Lazarous DF Unger EF, Epstein SE, Stine A, Arevalo JL, Chew EY, Quyyumi AA (2000) Basic Fibroblast Growth Factor in Patients With Intermittent Claudication: Results of a Phase I Trial. J Am Coll Cardiol 36:1239-1244

Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia AN, Hermiller JB, Hillegass WB, Rocha-Singh K, Moon TE, Whitehouse MJ, Annex BH (2002) Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet 359:2053–2058

Lee RJ, Springer ML, Blanco-Bose W, Shaw R, Ursell PC, Blau H (2000) VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 102: 898-901

Lefaucheur JP, Sebille A (1995) Basic fibroblast growth factor promotes in vivo muscle regeneration in murine muscular dystrophy. Neurosci Lett 202:121-124

Lefaucheur JP, Gjata B, Lafont H, Sebille A (1996) Angiogenic and inflammatory responses following skeletal muscle injury are altered by immune neutralization of endogenous basic fibroblast growth factor, insulin-like growth factor-I and transforming growth factor-1. J Neuroimmunol 70:37-44

Leiden JM (2000) Human gene therapy. The good, the bad, and the ugly. Circ Res 86:923-925

Le Quintrec JS, Le Quintrec JL (1991) Drug-induced myopathies. Baillieres Clin Rheumatol 5:21-38

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306-1309

Lochmuller H, Petrof BJ, Pari G, Larochelle N, Dodelet V, Wang Q, Allen C, Prescott S, Massie B, Nalbantoghu J, Karpati G (1996) Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. Gene Ther 3:706-716

Lu Y, Shansky J, Tatto MD, Ferland P, Wang X, Vandenburgh H (2001) Recombinant vascular endothelial growth factor secreted from tissue-engineered bioartificial muscle promotes localized angiogenesis. Circulation 104:594-599

Makinen K, Manninen H, Hedman M, Matsi P, Mussalo H, Alhava E, Yla-Herttuala S (2002) Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. Mol Ther 6:127–133

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55-60

Masaki I, Yonemitsu Y, Yamashita A, Sata S, Tanii M, Komori K, Nakagawa K, Hou X, Nagai Y, Hasegawa M, Sugimachi K, Sueishi K (2002) Angiogenic gene therapy for experimental critical limb ischemia: acceleration of limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. Circ Res 90:966-973

McGee GS, Davidson JM, Buckley A, Sommer A, Woodward SC, Aquino AM, Barbour R, Demetriou AA (1988) Recombinant basic fibroblast growth factor accelerates wound healing. J Sur Res 45:145-153

Melillo G, Scoccianti M, Kovesdi I, Safi J Jr, Riccioni T, Capogrossi MC (1997) Gene therapy for collateral vessel development. Cardiovasc Res 35:480-489

Milia AF, Salis MB, Stacca T, Pinna A, Madeddu P, Trevisani M, Geppetti P, Emanueli C (2002) Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia. Circ Res 91:346-352

Miller AD and Rosman GJ (1989) Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980-999

Molnar G, Ho MI, Schroedl NA (1996) Evidence for multiple satellite cell populations and a non-myogenic cell type that regulated differently in regenerating and growing skeletal muscle. Tissue Cell 28:547-556

Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L (1986) Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297–7301

Mooney DP, O'Reilly M, Gamelli RL (1990) Tumour necrosis factor and wound healing. Ann Surg 211:124-129

Morgan JE, Hoffman Ep, Partridge TA (1990) Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. J Cell Biol 111:2437-2449

Morgan JE, Pagel CN, Sherratt T, Partridge TA (1993) Long-term persistence and migration of myogenic cells injected into preirradiated muscles of mdx mice. J Neurol Sci 115:191-200

Mori H, Hyodo K, Tobita K, Chujo M, Shinozaki Y, Sugishita Y, Ando M (1994) Visualization of penetrating transmural arteries in situ by monochromatic synchroton radiation. Circulation 89:863-871

Morishita R, Sakaki M, Yamamoto K, Iguchi S, Aoki M, Yamasaki K, Matsumoto K, Nakamura T, Lawn R, Ogihara T, Kaneda Y (2002) Impairment of collateral formation in lipoprotein(a) transgenic mice: therapeutic angiogenesis induced by human hepatocyte growth factor gene. Circulation 105:1491-1496

Muhlhauser J, Jones M, Yamada I, Cirielli C, Lemarchand P, Gloe TR, Bewig B, Signoretti S, Crystal RG, Capogrossi MC (1996) Safety and efficacy of in vivo gene transfer into the porcine heart with replication-deficient, recombinant adenovirus vectors. Gene Ther 3:145–153

Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM (1998) Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 101:2567–2578

Nabel EG, Gordon D, Yang Z-Y, Xu L, San H, Plautz GE, Wu B-Y, Gao X, Huang L, Nabel GJ (1992) Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum Gene Ther 3:649-656

Nabel EG (2002) Stem cells combined with gene transfer for therapeutic vasculogenesis: magic bullets? Circulation 105:672-674

Neufeld G, Cohen T, Gengrinovitch S, Poltorak I (1999) Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13:9-22

Neumeister MW, Song Y-H, Mowlavi A, Suchy H, Mathur A (2001) Effects of liposome-mediated gene transfer of VEGF in ischemic rat gracilis muscle. Microsurgery 21:58-62

Ohara N, Koyama H, Miyata T, Hamada H, Miyatake SI, Akimoto M, Shigematsu H (2001) Adenovirus-mediated ex vivo gene transfer of basic fibroblast growth factor promotes collateral development in a rabbit model of hind limb ischemia. Gene Ther 8:837-845

Onimaru M, Yonemitsu Y, Tanii M, Nakagawa K, Masaki I, Okano S, Ishibashi H, Shirasuna K, Hasegawa M, Sueishi K (2002) Fibroblast growth factor-2 gene transfer can stimulate hepatocyte growth factor expression irrespective of hypoxia-mediated downregulation in ischemic limbs. Circ Res 91:923-930

Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM (2004) Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J Clin Invest 113:516-527

Palmer RM, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526

Palmer-Kazen U, Wariaro D, Luo F, Wahlberg E (2004) Vascular endothelial cell growth factor and fibroblast growth factor 2 expression in patients with critical limb ischemia. J Vasc Surg 39:621-628

Paoni NF, Peale F, Wang F, Errett-Baroncini C, Steinmetz H, Toy K, Bai W, Williams PM, Bunting S, Gerritsen ME, Powell-Braxton L (2002) Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice. Physiol Genomics 11:263-272

Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, Sessa WC (1999) Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival and interaction with other angiogenic growth factors. Lab Invest 79:213-223

Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845-848

Pouzet B, Vilquin JM, Hagege AA, Scorcin M, Emmanuel M, Fiszman M, Schwartz K, Menache P (2000) Intramyocardial transplantation of autologous myoblasts:can tissue processing be optimized? Circulation 102(Suppl III):III-210-III-215

Procopio WN, Pelavin PI, Lee WMF, Yeilding NM (1999) Angiopoietin-1 and -2 coiled-coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. J Biol Chem 277:30196-30201

Pu LQ, Sniderman AD, Brassard R, Lachapalle KJ, Graham AM, Lisbona R, Symes JF (1993) Enhanced revascularization of the ischemic limb by means of angiogenic therapy. Circulation 88:208-215

Pu LQ, Jackson S, Lachapelle KJ, Arekat Z, Graham AM, Lisbona R, Brassard R, Carpenter S, Symes JF (1994) A persistent hindlimb ischemia model in the rabbit. J Invest Surg 7:49–60

Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, Mytinger J, Cao B, Gates C, Wernig A, Huard J (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157:851-864

Qu Z, Huard J (2000) Matching host muscle and donor myoblasts for myosin heavy chain improves myoblast transfer therapy. Gene Ther 7:428-437

Rando TA, Blau HM (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol 125:1275-1287

Rando AT, Palvath GK, Blau HM (1995) The fate of myoblasts following transplantation into mature muscle. Experimental cell research 220:383-389

Rantanen J, Hurme T, Lukka R, Heino J, Kalimo H (1995) Satellite cell proliferation and the expression of myogenin and desmin in regenerating skeletal muscle: evidence for two different populations of satellite cells. Lab Invest 72:341-347

Risau W (1997) Mechanisms of angiogenesis. Nature 386:671-674

Rissanen TT, Markkanen JE, Arve K, Rutanen J, Kettunen MI, Vajanto I, Jauhiainen S, Cashion L, Gruchala M, Narvanen O, Taipale P, Kauppinen RA, Rubanyi GM, Yla-Herttuala S (2003) Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. FASEB J 17:100-102

Robinson CJ, Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. Journal of Cell Science 114:853-865

Roessler BJ, Davidson BL (1994) Direct plasmid mediated transfection of adult murine brain cells in vivo using cationic liposomes. Neurosci Lett 167:5-10

Rohovsky SA, Hirschi KK, D'Amore PA (1996) Growth factor effects on a model of vessel formation. Surg. Forum 47:390-391

Sato TN, Qin Y, Kozak CA, Audus KL (1993) Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA 90:9355-9358

Sato TN, Tozawa Y, Deutsch U, Wolburg-Bucholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376:70-74

Schainfeld RM, Isner JM (1999) Critical limb ischemia: nothing to give at the office? Ann Intern Med 130:442-444

Schaper W, Flameng W, Winkler B, Wuesten B, Türschmann W, Neugebauer G, Carl M, Pasyk S (1976) Quantification of collateral resistance in acute and chronic experimental occlusion in the dog. Cir Res 39:371-377

Schwartz B, Benoist C, Abdallah B, Scherman D, Behr JP, Demeneix BA (1995) Lipospermine-based gene transfer into the newborn mouse brain is optimized by a low lipospermine/DNA charge ratio. Hum Gene Ther 6:1515-1524

Schwartz RS, Stollar BD (1985) Origins of anti-DNA autoantibodies. J Clin Invest 75:321-327

Schultz E, McCormick KM (1994) Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol 123:213-257

Scorsin M, Hagege A, Vilquin JT, Fiszman M, Marotte F, Samuel JL, Rappaport L, Schwartz K, Menasche P (2000) Comparison of the effects of fetal cardiomyocyte and skeletal myoblast transplantation on postinfarction left ventricular function. J Thorac Cardiovasc Surg 119:1169–1175

Seifert FC, Banker M, Lane B, Bagge U, Anagnostopoulos CE (1985) An evaluation of resting arterial ischemia models in the rat hind limb. J Cardiovasc Surg 26:502-508

Sejersen T, Betsholtz C, Sjolund M, Heldin CH, Westermark B, Thyberg J (1986) Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein. Proc Natl Acad Sci U S A 83:6844-6848

Semenza GL (1999) Hypoxia inducible factor 1: master regulator of O_2 homeostasis. Curr Opin Gene. Dev, 8:588–594

Senger DR Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983-985

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood island formation and vasculogenesis in Flk-1-deficient mice. Nature 367:62-66

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5:519-524

Shimpo M, Ikeda U, Maeda Y, Takahashi M, Miyashita H, Mizukami H, Urabe M, Kume A, Takizawa T, Shibuya M, Ozawa K, Shimada K (2002) AAV-mediated VEGF gene transfer into skeletal muscle stimulates angiogenesis and improves blood flow in a rat hindlimb ischemia model. Cardiovasc Res 53:993-1001

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296-1299

Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845

Shyu KG, Chang H, Isner JM (2003a) Synergistic effect of angiopoitein-1 and vascular endothelial growth factor on neoangiogenesis in hypercholesterolemic rabbit model with acute hindlimb ischemia. Life Sci 73:563-579

Shyu KG, Chang H, Wang BW, Kuan P (2003b) Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 114:85-92

Silvestre JS, Levy BI (2002) Angiogenesis therapy in ischemic disease. Arch Mal Coeur Vaiss 95:189-96

Skuk D, Tremblay JP (2000) Progress in myoblast transplantation: a potential treatment of dystrophies. Microsc Res Tech 48:213-222

Smythe GM, Fan Y, Grounds MD (2000a) Enhanced migration and fusion of donor myoblasts in dystrophic and and normal host muscle. Muscle Nerve 23:560–574

Smythe GM, Grounds MD (2000b) Exposure to tissue culture conditions can adversely affect myoblast behaviors in vivo in whole muscle grafts: implications for myoblast transfer therapy. Cell Transplant 9:379-393

Smythe GM, Hodgetts SI, Grounds MD (2000c) Immunobiology and the future of myoblast transfer therapy. Mol Ther 1:303-313

Smythe GM, Grounds MD (2001a) Absence of MyoD increases donor myoblast migration into host muscle. Experimental Cell Research 267:267-274

Smythe GM, Hodgetts SI, Grounds MD (2001b) Problems and solutions in myoblasts transfer and therapy. J Cell Mol Med 5:33-47

Smythe GM, Lai MC, Grounds MD, Rakoczy PE (2002) Adeno-associated virus-mediated vascular endothelial growth factor gene therapy in skeletal muscle before transplantation promotes revascularization of regenerating muscle. Tissue Eng 8: 879-891

Springer ML, Chen AS, Kraft PE, Bednarski M, Blau H (1998) VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Molecular Cell 2:549-558

Springer ML, Ozawa CR, Banfi A, Kraft PE, Ip TK, Brazelton TR, Blau HM (2003) Localized arteriole formation directly adjacent to site of VEGF-induced angiogenesis in muscle. Mol Ther 7:441-449

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD (1996) Requisite role of Angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. Cell 87:1171-1180

Suzuki K, Brand NJ, Smolenski RT, Jayakumar J, Murtuza B, Yacoub MH (2000) Development of a novel method for cell transplantation through the coronary artery. Circulation 102(Suppl):III-359–III-364

Suzuki K, Murtuza B, Smolenski RT, Sammut LA, Suzuki N, Kaneda Y, Yacoub MH (2001) Cell transplantation for the treatment of acute myocardial infarction using VEGF-expressing skeletal myoblasts. Circulation 104:I207-I212

Tabata H, Silver M, Isner J (1997) Arterial gene transfer of aFGF for therapeutic angiogenesis in vivo: critical role of secretion signal in the use of naked DNA. Cardiovasc Res 35:470-479

Takeshita S, Pu LQ, Stein LA, Sniderman AD, Bunting S, Ferrara N, Isner JM, Symes JF (1994a) Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. Circulation 90:II228-II234

Takeshita S, Zheng LP, Brogi E, Kearney M, Pu L-Q, Bunting S, Ferrara N, Symes JF, Isner JM (1994b) Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J Clin Invest 93:662-670

Takeshita S, Rossow ST, Kearney M, Zheng LP, Bauters C, Bunting S, Ferrara N, Symes JF, Isner JM (1995) Time course of increased cellular proliferation in collateral arteries after administration of vascular endothelial growth factor in a rabbit model of lower limb vascular insufficiency. Am J Pathol 147:1649-1660

Takeshita S, Weir L, Chen D, Zheng LP, Riessen R, Bauters C, Symes JF, Ferrara N, Isner JM (1996) Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. Biochem Biophys Res Commun 227:628-635

Takeshita S, Isshiki T, Tanaka E, Eto K, Miyazawa Y, Tanaka A, Shinozaki Y, Hyodo K, Ando M, Kubota M, Tanioka K, Umetani K, Ochiai M, Sato T, Mori H, Miyashita H (1997) Use of synchroton radiation microangiography to assess development of small collateral arteries in a rat model of hindlimb ischemia. Circulation 95:805-808

Takeshita S, Isshiki T, Ochiai M, Eto K, Mori H, Tanaka E, Umetani K, Sato T (1998) Endothelium-dependent relaxation of collateral microvessels after intramuscular gene transfer of vascular endothelial growth factor in a rat model of hindlimb ischemia. Circulation 98:1261-1263

Taniyama Y, Morishita R, Aoki M, Nakagami H, Yamamoto K, Yamazaki K, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T (2001) Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hind limb ischemia models: preclinical study for treatment of peripheral arterial disease. Gene Ther 8:181-189

Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE (1998) Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med. 4:929–933

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophy Res Commun 187:1579-1586

Thompson RB, Emani SM, Davis BH, van den Bos EJ, Morimoto Y, Craig D, Glower D, Taylor DA (2003) Comparison of intracardiac cell transplantation: autologous skeletal myoblasts versus bone marrow cells. Circulation. 108:II264-71

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286:2511-2514

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6:460-463

Tomasi V, Manica F, Spisni E (1990) Polypeptide growth factors and angiogenesis. BioFactors 2:213-217

Torrente Y, El Fahime E, Caron NJ, Bresolin N, Tremblay JP (2000) Intramuscular migration of myoblasts transplanted after muscle pretreatment with metalloproteinases. Cell Transplant 9:539-550

Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, Passeri J, Horowitz JR, Symes JF, Isner JM (1996) Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation 94:3281-3290

Tsurumi Y, Kearney M, Chen D, Silver M, Takeshita S, Yang J, Symes JF, Isner JM (1997) Treatment of acute limb ischemia by intramuscular injection of vascular endothelial growth factor gene. Circulation 96:II-382-II-388

Tuder RM, Flook BE, Voelkel NF (1995) Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia: modulation of gene expression by nitric oxide. J Clin Invest 95:1798-1807

Urbanova D, Janda J, Mihova O, Linhart J (1974) Enzyme changes in the ischemia of skeletal muscle and the effect of physical conditioning: a histological study. Histochem J 6:147-153

Vale PR, Isner JM, Rosenfield K (2001) Therapeutic angiogenesis in critical limb and myocardial ischemia. J Interv Cardiol 14:511-528

Verma IM (2000) A tumultuous year for gene therapy. Mol Therapy 2:415-416

Vile RG, Tuszynski A, Castleden S (1996) Retroviral vectors: from laboratory tools to molecular medicine. Mol Biotechnol 5:139-158

Vincent KA, Shyu KG, Luo Y, Magner M, Tio RA, Jiang C, Goldberg MA, Akita GY, Gregory RJ, Isner JM (2000) Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. Circulation 102:2255-2261

Walder CE, Errett CJ, Bunting S, Lindquist P, Ogez JR, Heinsohn HG, Ferrara N, Thomas GR (1996) Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hind limb ischemia. J Cardiovasc Pharmacol 27:91-98

Watt DJ (1982) Factors which affect the fusion of allogeneic muscle precursor cells in vivo. Neuropathol Appl Neurobiol 8:135-147

Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu J-S, Isner JM (1998) Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of issue ischemia. Am J Pathol 153:381-394

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242-248

Yang HT, Deschenes MR, Ogilvie RW, Terjung RL (1996) Basic fibroblast growth factor increases collateral blood flow in rats with femoral arterial ligation. Circ Res 79:62-69

Yang HT, Feng Y, Allen LA, Protter A, Terjung RL (2000) Efficacy and specificity of bFGF increased collateral flow in experimental peripheral arterial insufficiency. Am J Physiol Heart Circ Physiol 278:H1966-1973

Yao SN, Kurachi K (1992) Expression of human factor IX in mice after injection of genetically modified myoblasts. Proc Natl Acad Sci USA 89:3357-3361

Yao SN, Smith KJ, Kurachi K (1994) Primary myoblast-mediated gene transfer: persistent expression of human factor IX in mice. Gene Ther 1:99-107

Young HE, Duplaa C, Young TM, Floyd JA, Reeves ML, Davis KH, Mancini GJ, Eaton ME, Hill JD, Thomas K, Austin T, Edwards C, Cuzzourt J, Parikh A, Groom J, Hudson J,

Black AC Jr (2001a) Clonogenic analysis reveals reserve stem cells in postnatal mammals: I. Pluripotent mesenchymal stem cells. Anat Rec 263:350-364

Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr (2001b) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264:51-62

Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE (2001) Cardiomyocyte grafting for cardiac repair: Graft cell death and anti-death strategies. J Mol Cell Cardiol 33:907–921

APPENDIX

Publications, Posters & Invited Seminar Presentations

- Autologous skeletal myoblasts transduced with a new adenoviral bicistronic vector for treatment of hind limb ischemia. *Muhammad I Niagara*, Husnain Kh Haider, Lei Ye, Valerie SW Koh, Yean T Lim, Kian K Poh, Ruowen Ge, Eugene KW Sim. Journal of Vascular Surgery (In Press).
- Therapeutic angiogenesis for treatment of peripheral vascular disease. *Niagara M Idris*, Husnain Kh Haider, Michelle WK Goh, Eugene KW Sim. Growth Factors (In press).
- Oral presentation on 'Angiogenic synergy between VEGF and Angiopoietin-1 together with myoblast therapy for the treatment of ischemically damaged hind limb. *Niagara M Idris*, Husnain Kh Haider, Yean Teng Lim, Kian Keong Poh, Ruowen Ge, Eugene KW Sim', American Heart Association Scientific Sessions 2004, New Orleans, Louisiana, USA, Nov 7-10, 2004.
- Represented Singapore Cardiac Society in the Young Investigator Award competition on 'Ex-vivo gene therapy using autologous myoblasts expressing VEGF and Angiopoietin-1 for the treatment of ischemically damaged limbs. *Niagara MI*, Haider KH, Ye L, Koh SW, Lim YT, Poh KK, Ge R, Sim EKW', 15th ASEAN Congress of Cardiology, Pattaya, Thailand, Oct 23–27, 2004.

- Poster presentation on 'Autologous Myoblasts Transduced With A Novel Bicistronic Adenoviral Vector Expressing VEGF And Angiopoietin-1 In A Rabbit Model of Hind Limb Ischemia. *Niagara Muhammad Idris*, Haider Khawaja Husnain, Ye Lei, Lim Yean Teng, Poh Kian Keong, Ge Ruowen, Sim Kwang Wei Eugene', National Healthcare Group (NHG) Annual Scientific Congress 2004, Raffles City Convention Centre, Singapore, Oct 9-11, 2004.
- Poster presentation on 'Enhanced Neovascularization Using Autologous Myoblasts as Carriers of Vascular Endothelial Growth Factor and Angiopoietin-1 in a Rabbit Model of Hindlimb Ischemia. *Niagara Muhammad Idris*, Haider Khawaja Husnain, Ye Lei, Lim Yean Teng, Poh Kian Keong, Ge Ruowen, Sim Kwang Wei Eugene', 8th National University of Singapore-National University Hospital Annual Scientific Meeting, Singapore, Oct 6-7, 2003.
- Abstract on 'Autologous Myoblasts Transduced With A Novel Bicistronic Vector Expressing VEGF and Angiopoietin-1 in a Rabbit Model of Hind Limb Ischemia. *Niagara M Idris*, Husnain Kh Haider, Lei Ye, Shujia Jiang, Wei S Koh, Teng T Lim, Keong K Poh, Eugene KW Sim', accepted for oral presentation in the 3rd European Association for Cardio-Thoracic Surgery/European Society of Thoracic Surgeons Joint Meeting, Leipzig, Germany, Sep 12-15, 2004.
- Oral presentation on 'Autologous myoblast transduced with a bicistronic vector encoding for human VEGF and Ang-1: A novel approach to treat ischemically damaged limbs. *Niagara Muhammad Idris*, Khawaja Husnain Haider, Ye Lei, Koh Seow Wei, Lim Yean Teng, Poh Kian Keong, Ge Ruowen, Eugene Sim

Kwang Wei', 5th Combined Annual Scientific Meeting Incorporating The 4th GSS-FOM Scientific Meeting, Clinical Research Centre, May 12-14, 2004.

- Oral presentation on 'Angiogenic Synergism Between Human VEGF and Angiopoietin-1 Together With Cell Therapy for the Treatment of Ischemically Damaged Hind Limb. *Niagara M Idris*, Husnain Kh Haider, Koh S Wei, Lim Y Teng, Poh Kian Keong, Henry Chan, Ge Ruowen, Eugene EK Sim', 12th Annual Meeting of the Asian Society for Cardiovascular Surgery, Istanbul, Turkey, Apr 19-22, 2004.
- Young Investigator Award oral presentation on 'Ex-vivo gene therapy using autologous myoblasts expressing VEGF and Angiopoietin-1 for the treatment of ischemically damaged limb. *Niagara MI*, Haider KH, Ye L, Koh SW, Lim YT, Poh KK, Ge R, Sim EKW', Singapore Cardiac Society 16th Annual Scientific Meeting, Ritz Carlton Millenia Singapore, Mar 21, 2004.
- Oral presentation on 'Enhanced Neovascularization Using Autologous Myoblasts as Carriers of VEGF165 in a Rabbit Model of Hind Limb Ischemia. *Niagara M Idris*, Khawaja H Haider, Ye Lei, Shujia Jiang, Lim Y Teng, Poh K Keong, Terrance Chua, Peter K Law, Eugene KW Sim', 16th Biennial Congress of Association of Thoracic and Cardiovascular Surgeons of Asia, Bangkok, Thailand, Nov 16-19, 2003.
- Poster presentation on 'Therapeutic Angiomyogenesis Using Autologous Myoblasts Carrying VEGF165 in a Rabbit Model of Hindlimb Ischaemia. *Niagara Muhammad Idris*, Khawaja Husnain Haider, Lei Ye, Shujia Jiang, Rufaihah Abdul Jalil, Yean Teng Lim, Kian Kong Poh, Terrance Chua, Peter K.

Law, Eugene Kwang Wei Sim', 7th National University of Singapore-National University Hospital Annual Scientific Meeting, Singapore, Oct 2-3, 2003.

- Angiomyogenesis in a rodent heart using myoblasts carrying $VEGF_{165}$. Ye Lei, Husnain Kh Haider, Jiang Shujia, *Niagara M Idris*, Rufaihah A Jalil, Peter K Law, Eugene KW Sim. International Journal of Medical Implants and Devices 2003; 1: 100-155.
- Myoblast therapy and angiogenesis: human myoblasts carrying human $VEGF_{165}$ for rehabilitation of injured heart. L Ye, Kh H Haider, SJ Jiang, LH Ling, *N Muhd Idris*, R Abd Jalil, PK Law, EKW Sim. 12th International Congress on Cardiovascular Pharmacotherapy, Barcelona, Spain, May 7-10, 2003.
- Ex-vivo delivery hVEGF165 for improved myocardial function in porcine heart model of chronic infarction. Ye Lei, Haider Kh Husnain, Shujia Jiang, *Niagara M Idris*, Peter K Law, Salim Aziz, Chua T, Wong P, Sim EKW. 11th Annual Meeting of the Asian Society for Cardiovascular Surgery, Kuala Lumpur, Malaysia, Feb 12-15, 2003.